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Seed Treating News from DU PONT

SUDAN GRASS AND FESCUE TESTS SHOW IMPORTANT BENEFITS FOR FARMERS WHEN SEED IS TREATED WITH "ARASAN"

More reports by farmers who conducted tests on their own farms in 1951 to compare grass and legume seed treated with "Arasan" seed disinfectant with untreated seed:

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E. M. Mitchell of Youngsville, planted Kentucky 31 Fescue in one of his fields on March 27. Part of the seed was treated with "Arasan" at the recommended rate and part of the seed was not treated. On May 15 the field was checked to determine what difference treating made.

Mr. Mitchell wrote: "I found a 30% better stand in the treated portion of the field, and, also, the fescue was approximately 2½ inchest taller. I feel that it pays to treat grass and legume seed."

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R. S. Quisenberry at Timberlake Farm, Frisco, Texas, planted Sweet Sudan—part treated with "Arasan" and part untreated. He found a 31% better stand in the treated portion of the field. When viewed from a distance, the plants appeared about the same, but, when plants from each part of the field were dug up and placed side by side, the plants produced from treated seed were approximately 50% longer and thicker.

These tests are convincing evidence that farmers benefit from this new and important agricultural practice. For an up-to-the-minute report of what is happening in grass and legume seed treating, write to Du Pont and ask for A-2511, "Grass and Legume Seed Booklet."

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A NEW SPECIES OF PERIDERMIUM ON VIRGINIA PINE[1]

George H. Hepting and George B. Cummins[2]

A new *Peridermium* occurring on stems of *Pinus virginiana* Mill. in North Carolina, Tennessee, Virginia, and West Virginia is reported in this paper. The rust is distinctive in that the aecia are columnar and mostly nonconfluent, and occur scattered singly over the sporulating area (Fig. 1, A, C). Moreover, the infected areas are not deformed, and not enlarged or only slightly enlarged, and, in many cases observed, the twigs and branches beyond the encircling lesions appeared healthy and normal. Killing is often slow, sometimes taking place years after aecia have erupted over the complete circumference.

The first collection was made near Waterville Lake, north of Waynesville, N. C., in April 1948, by W. A. Stegall of the Division of Blister Rust Control, U. S. Bureau of Entomology and Plant Quarantine. Since that time, additional collections have been made near the original site, as well as in Mitchell County, N. C.; Wythe, Alleghany, Montgomery, and Pulaski counties, Virginia; Greenbrier County, West Virginia, and Morgan and Unicoi counties, Tennessee. Careful field observations in North Carolina and Virginia have failed to reveal the presence of a telial stage. Since the rust has some similarities to *Cronartium comptoniae* Arth., a special search was made for sweetfern and sweetgale, the telial hosts of *C. comptoniae*. Neither of these plants was found anywhere near the rusted pines.

Inoculation experiments, made in the field by E. R. Roth and E. R. Toole of the Division of Forest Pathology, substantiate the belief that this *Peridermium* is not a variant of *Cronartium comptoniae*. Aeciospores were sprayed onto new leaves and shoots of sweetfern and onto new growth of Virginia pine during the period May 2 to May 9, 1950, near Asheville, N. C. The inoculated portions of the plants were covered with celluloid cones for 48 to 72 hr. and the plants were located in shade. No infection resulted on either sweetfern or pine. These results are not regarded as conclusive, particularly the inoculations made on pine. Additional trials and at least three years of observation are required to substantiate more fully the possibilities of pine-to-pine infection.

Because of the morphological characteristics of the fungus, the type of lesion produced, and the negative results of inoculations, it is believed that this rust represents an undescribed species. The telial host, if there is one, is unknown. A description of the fungus follows.

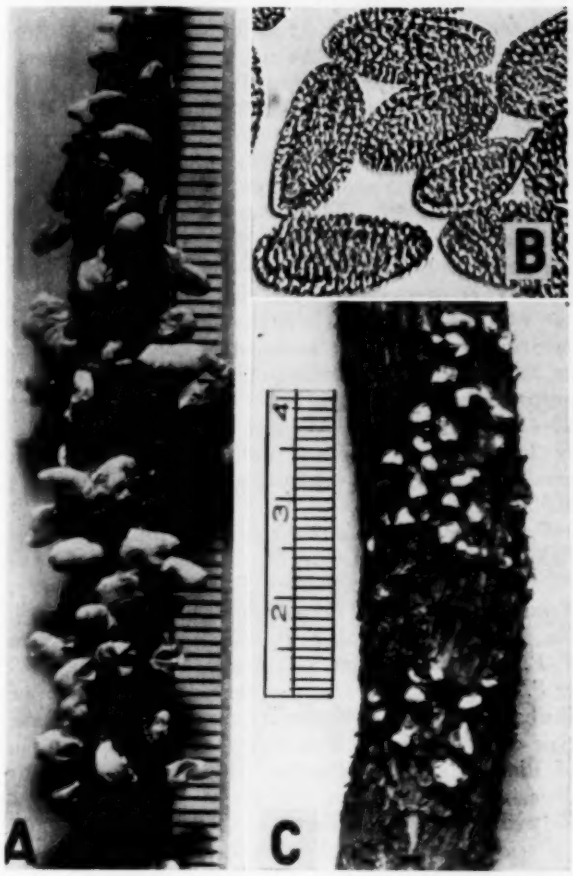

FIG. 1. *Peridermium appalachianum*. A. Aecia, showing the nearly uniform distribution and the columnar form. Intrasoral filaments are scarce or lacking. (× 2, the scale in mm.) B. The long narrow aeciospores with uniformly verrucose wall characteristic of this rust. (× 800) C. Immature aecia breaking through the bark. Note lack of swelling. (× 1.5, the scale in mm.)

Peridermium (*Cronartium*) **appalachianum** n. sp.

Spermagoniis non visis. Aeciis (Fig. 1, A) ramicolis, tumores haud efficientibus, e cortice erumpentibus, plus minusve aequaliters sparsis, magnis, 1.5–5.0 mm. latis, 2.0–7.0 mm. altis, cylindraceis vel linguaeformibus, flavo-aurantiacis, superne vel plerumque ad latera disrumpentibus, e seriebus 1–2 cellularum constructis, cellulis peridio ellipsoideis, rhomboideo-ellipsoideis, vel subfusiformibus, 16–23 × 40–80 μ, membrana 3–4 μ crassa, minute rugoso-verrucosa; filamentis intersporarum nullis vel perpaucis; aeciosporia ellipsoideis vel oblongo-ellipsoideis, raro late ellipsoideis

[1] Accepted for publication September 15, 1951.

[2] Respectively, Senior Pathologist, Division of Forest Pathology, Bureau of Plant Industry, Soils, and Agricultural Engineering, U. S. Department of Agriculture, in cooperation with the Southeastern Forest Experiment Station; and Associate Botanist, Purdue University Agricultural Experiment Station and Agent, Division of Mycology and Disease Survey, U. S. Department of Agriculture. Journal Paper No. 538 of the Purdue University Agricultural Experiment Station.

vel fusiformibus, (11–) 13–19 (–22) × (19–) 29–43 (–53) μ; membrana hyalina, uniformiter 2–3 μ crassa, moderate et uniformiter verrucosa.

On *Pinus virginiana*, in North Carolina, Tennessee, Virginia, and West Virginia, U. S. A.

Spermagonia uncertain, not found by sectioning or field observation. Aecia (Fig. 1, A, C) on branches, fruiting from April to August, causing little hypertrophy and no distortion, usually arising singly in the sporulating area, orange when fresh, pale yellow when dry, blister like at first but becoming cylindrical or somewhat flattened laterally and tonguelike, 1.5–5.0 mm. wide, 2.0–7.0 mm. high, mostly opening by longitudinal slits, without or only occasionally with a few intrasoral filaments; peridium firm, the peridial cells variable but those midway on the sides mostly long ellipsoid or narrowly diamond-shaped, 16–23 × 40–80μ, the wall 3–4μ thick, finely rugose-verrucose, often in a labyrinthiform pattern; aeciospores (Fig. 1, B) mostly long ellipsoid or oblong-ellipsoid, rarely broadly ellipsoid or fusiform, (11–) 13–19 (–22) × (19–) 29–43 (–53) μ or rarely longer, wall hyaline, uniformly 2–3μ thick, closely and uniformly verrucose with small, flat, irregularly shaped warts; germ pores not visible.

On branches of *Pinus virginiana* Mill., Waterville Lake, west of Waynesville, N. C., April 1948, *Stegall F. P. 90356* (type); Pollers Ford, Morgan County, Tenn., May 20, 1949, *Stegall F. P. 90368*; Speedwell, Va., April 1949, *Stegall F. P. 90366*; April 1950, *Roth F. P. 90442*; Covington, Va., August 21, 1950, *Rodamer F. P. 90603*. Type specimen deposited in the Arthur Herbarium, Purdue University, and portions of the type collection in the Mycological Collections, Bureau of Plant Industry, Beltsville, Md. Other collections deposited in Herbarium, Division of Forest Pathology, Beltsville, Md., and Asheville, N. C.

Peridermium appalachianum has somewhat the appearance of the aecia of *Cronartium comptoniae*. It is readily separable, however, because of the long nonconfluent aecia, the scarcity or complete absence of intrasoral filaments, and the long, narrow, and uniformly verrucose aeciospores. The lack of hypertrophy, characteristic of this species, is sometimes approached by *C. comptoniae* although the latter usually causes fusiform enlargements. No European or Asiatic species has similar aeciospores.

Peridermium appalachianum is morphologically similar, in some respects, to *P. filamentosum* Peck, which is treated by Arthur[3] as one of the aecial stages of *Cronartium coleosporioides* (Diet. & Holw.) Arth. The size, shape, manner of dehiscence, and distribution of the aecia are similar and no hypertrophy of the host is caused by either species. We have no evidence that the peridia of *P. appalachianum* persist through the winter as do those of *P. filamentosum*. While an occasional filament, pendent from the dome of the peridium, occurs in *P. appalachianum*, most aecia have none. Filaments, running the length of the aecia, are abundant in *P. filamentosum* and are present even after overwintering. As to aeciospores, *P. appalachianum* has consistently longer and narrower spores than does *P. filamentosum*. The average in *P. appalachianum* is 15 × 34μ as against 21 × 29μ in *P. filamentosum*. The thickness of the wall and the degree of sculpturing are similar in both species except that there is some tendency toward a smooth basal or lateral area on the spores of *P. filamentosum*, whereas the sculpturing is uniform in *P. appalachianum*. *P. stalactiforme* Arth. & Kern, also referred to as *C. coleosporioides* by Arthur,[3] has shorter, more robust aeciospores with much thicker walls and a constantly smooth basal area.

Field observations.—Some observations on canker abundance and effects were made at Speedwell and Covington, Va., on April 19, 1951. At Speedwell sapling and pole-sized trees were observed and tallied along a road for a distance of 1/10 mile. Fifty-one cankers were found, of which 65 per cent were alive and producing mature aecia, and 35 per cent were dead, having killed a branch or main stem. Two trees, one 2 in. and the other 3 in. in diameter, breast high, had been killed by the rust. The largest number of cankers on a single tree was 18, on a large tree. The oldest cankers originated about 1936, with some originating almost every year from 1940 to 1949.

At Covington, a 1/20-acre plot was laid out in an infected sapling stand. Of the 42 trees tallied, 1 in. or over, d.b.h., 29 per cent bore cankers somewhere on the tree, 10 per cent bore trunk cankers, and many branches but no trees had been killed. Of 53 smaller trees, 8 per cent were cankered and 4 per cent had been killed. The oldest cankers tallied originated about 1942, with some fruiting cankers appearing on the wood of each year up to 1949. Some large trunk cankers were found within a few feet of the ground on trees as large as 6 and 7 in. d.b.h.

This rust can work its way down a branch for a foot or more, often entering the trunk. Its action is more like that of *Cronartium ribicola* than like that of *C. cerebrum* or *C. fusiforme*. A girdling bark lesion is formed, without the wood-cell hypertrophy common in the case of the other southern pine stem rusts. The part of the stem or branch beyond the canker dies from two to several years after the fungus has encircled the stem. If the fungus is a recent introduction, it could be viewed with some alarm. Its pathogenicity to other hard pines is not known. If it is a long-established or native organism it will probably prove of little importance, since damage so far has been slight.

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Asheville, North Carolina
and
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Lafayette, Indiana

[3] Arthur, J. C. Manual of the rusts in United States and Canada. 438 pp. 1934. Lafayette, Ind.

FURTHER STUDIES ON ENVIRONMENT AFTER SEEDLING EMERGENCE AND OTHER FACTORS THAT INFLUENCE THE INCIDENCE OF BARLEY COVERED SMUT[1]

V. F. Tapke[2]

Escape from infection often obscures the true susceptibility of small grain in varieties and hybrids that are being bred for resistance to smut. Previous investigations on the infection of barley with covered smut (*Ustilago hordei* (Pers.) Lagerh.) have shown that method of artificial seed inoculation, soil conditions during seedling emergence, and post-emergence environment each may influence the percentage of infected plants (5). The writer has reported striking effects of low temperature after seedling emergence in the reduction of covered smut of barley (*U. hordei*), nigra loose smut of barley (*U. nigra*), and loose smut of oats (*U. avenae*) (2, 3, 4). The present study was undertaken to determine the interaction of low temperature after seedling emergence with method of artificial seed inoculation and soil temperature during emergence, and the relative importance of each of the three factors. Reed (1) and recently the writer (5) have reviewed the literature on influence of various post-emergence conditions on incidence of the cereal smuts.

In a test at Beltsville, Maryland, in 1944, the combined influences of each of five soil temperatures during seedling emergence (40°, 50°, 60°, 70°, and 80° F.) and field or greenhouse conditions after emergence in February were studied. Smut-free seed of Hannchen (C. I. 531) and Pannier (C. I. 1330) spring barleys was inoculated in mid-January with covered smut (*Ustilago hordei*) by the spore-suspension method (6). The inoculated seed was air-dried to its initial weight and then stored in sealed glass jars at 30°–32° F. Since soil temperatures materially affect the rate of seedling emergence, the inoculated seed was sown on different dates as determined by preliminary trials, so that all seedlings would be approximately 1 in. above ground on the same day in mid-February when the different post-emergence treatments were started. At soil temperatures of 40°, 50°, 60°, 70°, and 80° F., seed sown 1 in. deep emerged in approximately 28, 12, 6, 5, and 4 days, respectively; thus the 40° flats were sown 28 days before the date selected for emergence and the other flats at the indicated intervals thereafter. Each of the 20 combinations of two varieties, five soil temperatures, and two post-emergence environments was replicated in three flats, each 12 by 24 in. by 6 in. deep. The seeds, 100 per flat, were sown uniformly 1 in. deep in soil, the surface of which was 1 in. below the top of the flat. All soil was stored in moisture-proof galvanized iron cans to prevent a change in moisture content between seedings. Immediately after seeding, the flats were placed in large insulated rooms with accurately controlled temperatures and covered tightly with heavy moisture-proof paper to prevent drying of the soil during germination. Upon seedling emergence in mid-February, 1944, some flats were transferred to a temperate greenhouse and some to outdoor cold-frames covered with glass sash, which was raised gradually and finally removed after one week. The outdoor plants suffered no reduction in stand due to winter killing. For each of the items involved, the three flats of each treatment produced approximately 250 to 275 plants.

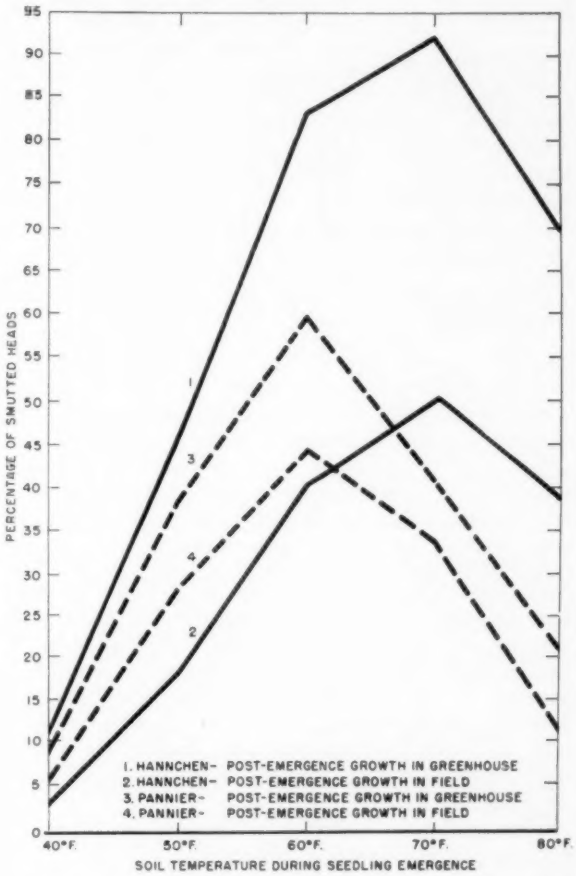

FIG. 1. Covered smut in Hannchen and Pannier barleys, as influenced by soil temperature during seedling emergence and by exposure of the plants after seedling emergence to outdoor conditions or temperate greenhouse conditions, from February to heading in April and May, 1944.

The data (Fig. 1) show that both soil germination temperature and conditions after seedling emergence affect the incidence of covered smut in barley. Moderate temperatures both before and after emergence

[1] Accepted for publication October 5, 1951.
[2] Plant Industry Station, Beltsville, Maryland.

proved most favorable for infection. Hannchen and Pannier responded somewhat differently.

In a concluding study in 1945 only the Hannchen variety was tested by two methods of seed inoculation, spore-suspension and superficial dusting (6). In all other procedures the techniques of the 1944 experiment were used again in 1945. The data from the 20 different combinations of two methods of seed-inoculation, five soil temperatures during seedling emergence, and two different environments after seedling emergence are in table 1; each smut percentage is based on 254 to 278 plants.

TABLE 1.—*Influence of method of seed inoculation, soil temperature during seedling emergence, and post-emergence environment on the incidence of covered smut in Hannchen barley*

Soil temperature during seedling emergence	Percentage of smutted plants from post-emergence growth (February-May, 1945)			
	Greenhouse		Field	
	A[a]	B[a]	A	B
40° F.	6.3	17.9	0.0	4.6
50	11.3	48.6	6.4	13.4
60	16.6	55.1	8.5	27.0
70	33.5	71.3	19.8	40.4
80	12.2	44.8	6.7	20.8
Average	16.0	47.6	8.1	21.1

[a] A—spore dust inoculation; B—aqueous spore-suspension inoculation.

The different treatments produced from none to 71.3 per cent smutted plants. The effect of the different soil temperatures during seedling emergence was pronounced. The average percentage of smutted plants from seed inoculated by the spore-suspension method was approximately three times that of plants from spore-dusted seed, while plants that grew in the greenhouse after emergence were approximately twice as heavily smutted, on the average, as those grown outdoors after emergence. This information may prove useful in tests in which a full expression of susceptibility of varieties and hybrids is sought.

PLANT INDUSTRY STATION
BELTSVILLE, MARYLAND

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A PHYTOPATHOLOGICAL NOTE

Reflected Light as an Aid in the Keitt Method of Spore and Hyphal Tip Isolations. R. S. COX.[1] A modified procedure of the Keitt method[2] has been used successfully by the writer for routine single-spore and single-hyphal-tip isolations. Necessary equipment consists of: (1) a concentrated light source as supplied by most microscope lamps; (2) a microscope with all objectives removed save that of the lowest magnification; (3) a platinum loop and a spatula as described by Keitt.[2]

For single-spore isolations, a dilute spore suspension is prepared and sown on solidified agar.[3] Using the low power objective of the microscope, a well-isolated spore is located in the opened petri plate. Diaphragm apertures of microscope lamp and condenser are adjusted so that the light beam reflected through the agar is reduced in outline to an area slightly larger than that of the spore. Without disturbing the petri plate, the microscope objective is then raised and swung to one side.

While sighting down the side of the microscope barrel, an impression is made around the spore by carefully lowering a flamed platinum loop until it presses against the agar surrounding the circumference of the light beam and cuts into the agar to a desired depth (approximately 1 mm.). The field is examined again under low power to determine whether the spore was delimited. The delimited portion of the agar is then lifted out with a spatula and placed either in a sterile petri plate or at the tip of an agar slant for further observation.[1,2] Little contamination occurs when working under still atmospheric conditions.

This technique is effective for making single-hyphal-tip isolations of certain rapid-growing fungi.—Dept. of Plant Pathology, Univ. of Del. Agr. Exp. Sta., Newark, Delaware.

[1] Published as Miscellaneous Paper No. 133 with the approval of the Director of the Delaware Agricultural Experiment Station. Contribution No. 41 of the Department of Plant Pathology.

[2] Keitt, G. W. Simple technique for isolating single-spore strains of certain types of fungi. Phytopath. 5: 266-269. 1915.

[3] Ezekiel, Walter N. Modified procedure with the Keitt single-spore method. Phytopath. 20: 583-586. 1930.

PHYSIOLOGICAL PROPERTIES OF FUSARIUM LYCOPERSICI AND FUSARIUM VASINFECTUM[1]

Oldrich K. Sebek[2]

Interest in Fusaria generally centers around their ability 1) to cause plant diseases such as destructive blight of cereal crops, wilts, and root rots of field and vegetable crops, and disorders of many ornamental and woody plants; 2) to bring about alcoholic fermentation; 3) to amass large quantities of fat within mycelial cells; and 4) to produce antibiotics.

Characteristic pigment formation by certain species of the genus *Fusarium* was studied in different laboratories; some of the pigments were isolated and their structures determined. In recent years Nord and his students synthesized some of these pigments and discovered that they have definite physiological functions and can hardly be considered as mere waste products. In a series of papers (1, 4, 6, 7, 13, 14) it was demonstrated that Fusarium pigments are able to play an active part in enzymatic reactions of the cell, and their participation as hydrogen carriers in the formation of fats from carbohydrates has been suggested (1). It was shown that increased lipide formation and a higher fat coefficient were accompanied by higher pigment production in the mycelium of *F. solani* D_2 purple. The amount of fat and of pigment increased with increasing concentration of glucose, and maximum fat formation coincided with maximum pigment production in *F. lycopersici* (6).

When Fusaria are maintained under laboratory conditions, the formation of pigments is not a consistent property; on transplantation, the quantity of pigment elaborated may decrease or even disappear completely. In a study of the function of lycopersin in the conversion of carbohydrate into fat by Fusaria, conditions were determined under which the ability of the molds under investigation to elaborate pigments was not attenuated and even could be stimulated. The present communication reports some of the results obtained.

EXPERIMENTAL WORK.—Two species of Fusaria were used in this study: 1) *F. lycopersici* R-5-6, obtained from Dr. S. P. Doolittle, U. S. Department of Agriculture, Beltsville, Maryland, and 2) *F. vasinfectum* F-273, from Dr. William J. Robbins, The New York Botanical Garden, New York 58, New York. The stock cultures were maintained on potato-dextrose agar and on malt agar.

Under suitable conditions each organism produced a pigment which was deep red in the lower pH ranges and blue when the pH was close to neutrality. On the basis of the same color reactions and an identical absorption spectrum, it was concluded that both species elaborated the same pigment (3). Since it was first isolated from *F. lycopersici*, the name lycopersin was assigned to it (2). It is a dihydroxy, dimethoxy compound, containing a phenyl radical. Microanalytical elemental data on the compound, and its derivatives, indicate the formula $C_{20}H_{15}O_8$ (or an integral multiple thereof). It is insoluble in most of the usual organic solvents.

F. lycopersici grew well but was only slightly pigmented on malt agar and potato-dextrose agar. A heavy coat of fluffy mycelium with no pigmentation appeared on potato plugs. *F. vasinfectum* produced a heavy growth of well-pigmented mycelium on malt agar and on potato-dextrose agar; although luxuriant mycelium was observed on potato plugs, pigmentation was slight. On nutrient agar both strains grew very poorly and elaborated no pigment.

The pigment was located in the mycelium and distributed discretely in the cells. It was also found in the chlamydospores. Its deposition was not observed in the free conidia which were formed and released profusely on both pigmented and nonpigmented mycelia. The pigment did not diffuse into the nutrient media.

For the experiments, 125- to 250-ml. Erlenmeyer flasks were used, containing 25–50 ml. of medium. Inoculations were made with 0.5- to 1.0-ml. suspensions of spores washed from the stock cultures with sterile water. Solid mats on liquid media usually developed in 3–5 days. A modified Raulin-Thom medium[3] was found suitable for cultivation. Although both species grew well in Czapek-Dox medium, they failed completely to produce pigment.

When the molds were allowed to grow at different temperatures on glucose media, change of temperature affected the rate of growth only (Table 1); pigment appeared in all instances only after a heavy mycelial pad had been formed.

An incubation temperature of 27° C. was used henceforth.

In order to determine the influence of pH on spore germination, growth, and formation of pigment, Raulin-Thom medium was supplemented with 5 per cent glu-

[1]Accepted for publication October 8, 1951.

Communication No. 000. The work was carried out under the auspices of the Office of Naval Research and was supported by a grant of the F. G. Cottrell Fund of the Research Corporation, New York, N. Y.

[2]Present address: Department of Bacteriology, The Ohio State University, Columbus 10, Ohio. The author expresses his indebtedness to Professor F. F. Nord for helpful counsel in the course of this investigation.

[3]This medium consisted of 4.0 gm. tartaric acid, 4.0 gm. ammonium tartrate, 0.6 gm. K_2CO_3, 0.6 gm. $(NH_4)_2HPO_4$, 0.4 gm. $MgCO_3$, 0.25 gm. $(NH_4)_2SO_4$, 0.07 gm. $ZnSO_4.7H_2O$, 0.07 gm. $FeSO_4.7H_2O$, and 1,000 ml. distilled water. Solutions were supplemented with carbohydrates or other carbon sources.

TABLE 1.—*Growth of Fusarium vasinfectum in 50 ml. of Raulin-Thom medium (10 per cent glucose; initial pH 3.4; 250-ml. Erlenmeyer flasks)*

°C.	Mycelium (mgm./50 ml.) and pH after					
	12 days		21 days		28 days	
13	65	3.2	214	2.5	228	2.6
20	474	2.7	463	2.3	426	2.2
27	368	2.6	397	2.3	465	2.3
32	232	2.8	380	2.5	391	2.4
37	No growth					

cose and adjusted to various initial pH values. The results after 14 days are in table 2.

TABLE 2.—*Relation of H-ion concentration to growth of two species of Fusarium in Raulin-Thom medium plus 5 per cent glucose after 14 days*

Organism	Initial pH	Final pH	Mycelium (mgm./25 ml. medium
F. lycopersici	2.0	2.0	0
	2.8	2.7	225
	3.3	2.4	349
	5.5	6.9	382
	7.4	7.2	561
F. vasinfectum	1.4	1.4	0
	1.9	1.9	2
	2.3	2.5	48
	2.7	2.1	62
	3.2	2.1	109
	3.7	3.0	397
	5.9	7.4	401
	6.9	7.5	431
	8.3	7.5	379

Growth occurred within a broad pH range and this substantially confirms the results of Webb (12) and of Sherwood (9), who obtained a light growth of *F. lycopersici* at pH 2.2 but no spore germination at pH 1.8. The most noticeable fact in our work, however, was that maximum pigment was produced by both species within the pH range of 3.2–3.7 and a fair quantity between pH 2.7 and 4.6. Dependence on the acidity of the medium was more critical with *F. vasinfectum* than with *F. lycopersici*.

The normal metabolism of both species was characterized by a steady increase of mycelial weight and a change in pH. At the beginning of the dissimilation, acids were formed which markedly lowered the pH (e.g., to 1.8). Such a high acidity was reached when a low carbohydrate concentration was used (about 1 per cent). At that time, the carbohydrate was generally exhausted, and the acids formed were the only carbon source. They disappeared as further oxidation proceeded; the pH rose and usually reached the alkaline side; pH 8.7 was the highest value observed. The alkalinity was due to the formation of ammonia, which was detected when either ammonium salts, nitrite, nitrate, or peptone was the sole nitrogen source. When higher carbohydrate levels were used (3–20 per cent), the pH fell and remained constant at 1.8–2.7. Representative pH values are recorded in tables 1–3. This state may represent the "isometabolic point" as defined by Sideris (10): it is the initial pH value of the nutrient solution which may be changed slightly or not at all by the reaction of the metabolic products, or toward which the final pH in other media is pointing during the growth; this point depends primarily on the ratio of available carbohydrate to protein which determines the ratio of organic acids to basic metabolic products, and on the resistance of the organism to the effects of its own metabolic products. From our experiments it can be concluded that the concentration of carbohydrate was critical; when the carbohydrate was available in excess, it was utilized easily and the acids formed from it then remained unaffected.

It has been stated repeatedly that Fusaria possess an extreme variability in their morphological and physiological properties, including virulence and certain cultural characteristics, especially pigmentation (11). Sherbakoff mentioned (8) that media rich in glucose were responsible for an increase in the amount of pigment and the density of color produced by *F. arcuosporum* or *F. angustum*. This observation was also made with *F. lycopersici* (6). When *F. vasinfectum* was grown in Raulin-Thom medium containing increasing concentrations of glucose or xylose, the results in table 3 were obtained.

TABLE 3.—*Growth of F. vasinfectum in increasing concentrations of glucose and xylose; 21 days' incubation in Raulin-Thom medium of initial pH 3.1–3.3*

Sugar (gm./100 ml.)	Mycelium (mgm./25 ml.) and final pH			
	Glucose		Xylose	
1.0	68	7.2	89	7.2
2.5	137	1.9	123	1.8
5.0	135	1.8	122	1.8
8.5	138	1.9	120	1.8
10.0	177	1.8	110	1.8
15.0	159	1.9	107	1.8
20.0	112	2.1	77	2.6
30.0	83	1.9	No growth	

Maximum amounts of mycelium were obtained at concentrations of 10 per cent glucose and 2.5–5.0 per cent xylose. Maximum pigment formation occurred at a sugar concentration slightly below that optimal for maximum growth.

In order to find a suitable nutrient medium which would give rise to the formation of the highest possible quantity of lycopersin in both species under investigation, various carbon sources were tested. Raulin-Thom medium was supplemented with polyhydric alcohols, sugars, and acids, inoculated, and incubated for 21 days. The amount of pigment produced and the amount of growth are in table 4. The results of this experiment also revealed information on the possible mechanism of pigment formation.

TABLE 4.—*Growth and pigment production after 21 days by (A) F. lycopersici and (B) F. vasinfectum in Raulin-Thom medium of initial pH 3.0–3.3 supplemented with selected C compounds. Acids neutralized with NaOH prior to use*

C source	Pigment[a] A	Pigment[a] B	Growth
Concentration, 0.1M			
Acetic acid	0	0	None
Propionic acid	0	0	None
Butyric acid	0	0	None
Lactic acid	0	0	None
Glycolic acid	0	0	Poor
Glyoxylic acid	0	0	Poor
Malonic acid	0	0	None
Oxalic acid	1	0	Heavy
Calcium gluconate	1	1	Heavy
Succinic acid	1	1	Moderate
Fumaric acid	2	1	Heavy
Malic acid	2	1	Heavy
Itaconic acid	0	0	Poor
Ethanol	3	4	Moderate
Glycerol	2	2	Poor
Sorbitol	3	3	Heavy
Dulcitol	3	3	Heavy
Concentration, 5 per cent			
Xylose	5	5	Heavy
Glucose	5	5	Heavy
Galactose	5	5	Heavy
Sorbose	5	5	Heavy
Sucrose	4	4	Heavy
Cellobiose	4	4	Heavy
Lactose	1	1	Heavy
Starch	4	4	Heavy
meso-Inositol	6	6	Heavy
Raulin-Thom	0	0	Moderate

[a] Numbers indicate the quantity of pigment formed: 0—none; 1—slight; 2—moderate; 3—fair; 4—pronounced; 5—heavy; 6—very heavy.

Fatty acids prevented the growth of both species completely. In the medium supplemented with lactic or malonic acids, the molds also failed to grow. No pigment was formed in the medium containing glycolic, glyoxylic, or itaconic acid. Starch provided a substrate both for growth and formation of pigment. The appearance of reducing sugars and a negative iodine test indicated the presence of amylases. When alcohols or sugars were supplied, growth was normal and the quantity of pigment significant in all cases, except in the lactose medium where only a slight pigmentation occurred after a prolonged incubation (14 or more days). Results similar to those in table 4 also were obtained with replacement cultures: both species were grown in Raulin-Thom medium plus 5 per cent lactose at pH 3.1–3.3 for 7 days. During that period nonpigmented mats developed. Nutritive liquids then were poured out of flasks, mycelial pads were washed with sterile water, and sterile solutions of different compounds as indicated above were added. The appearance of pigment occurred in the same instances as recorded in table 4.

The effect of *meso*-inositol was striking. It supported growth well, and gave rise to highly pigmented mycelia in acid medium. However, when the initial pH was close to neutrality, no pigment appeared. Thus, by a combination of these observations, it was possible to devise experimental conditions in such a way that graded amounts of pigment were produced in the mycelia, and the effect of lycopersin on the composition of the lipides produced by *F. vasinfectum* could be studied. An example of this procedure is given in table 5.

TABLE 5.—*Effect of meso-inositol and lactose on the composition of fats and on the pigment formation by F. vasinfectum grown in Raulin-Thom medium for 21 days* [a]

Datum	Carbon source: Inositol, 2.5 per cent	Inositol, 1.25 per cent, plus lactose, 1.25 per cent	Lactose, 2.5 per cent
Initial pH	3.3	3.3	7.0
Mycelium (gm./liter)	6.08	7.25	1.31
Total lipides (per cent)	19.8	15.1	21.3
Sterols (per cent)	1.17	0.83	1.01
Iodine value	104.0	152.0	165.0
Pigment in mycelium (mgm./gm.)	3.48	2.00	0.00

[a] The analytical methods employed have been described previously (3).

In the absence of lycopersin, a less saturated fat is produced. Ultraviolet absorption spectra of isomerized oils showed that the formation of linolenic acid was responsible for this effect. The mechanism and function of lycopersin in this phenomenon have been discussed (3).

It has been reported that Fusaria require certain heavy metals for the normal development of pigment (5). Young and Bennett stated earlier (15) that a striking color formation appeared in *F. radicicola* which was colorless in solutions containing potassium but turned pink in media containing calcium. Our results showed that zinc ions (as $ZnSO_4$), when present in trace amounts, stimulated the production of mycelial mass (e.g., 4 p.p.m. doubled the weight as compared with the control without Zn^{++}), but had no effect on the production of pigment (Table 6).

TABLE 6.—*Effect of Zn on growth of F. lycopersici and F. vasinfectum incubated in 50 ml. of modified Raulin-Thom medium in 250-ml. Erlenmeyer flasks, determined after 28 days*

Zn (p.p.m.)	Mycelium (mgm./50 ml.) *F. lycopersici*	Mycelium (mgm./50 ml.) *F. vasinfectum*
0	148	167
4	335	349
16	359	361
25	350	359
50	331	327
100	315	362

When cupric ions (as $CuSO_4$) were supplied to the medium in the concentration of 2.5×10^{-5} gm./liter, a heavily pigmented mycelium of *F. lycopersici* was produced in Raulin-Thom medium with 5 per cent glucose. Furthermore, when the nutrient medium underneath the unpigmented mats of *F. lycopersici* was replaced with aqueous solutions of compounds which gave rise to pigment, as indicated in table 4, and was adjusted to pH 3.0–3.3, pigment appeared in every case, even in the lactose solution, unlike the cultures of *F. vasinfectum*.

Thus, by a combination of these factors, pigment formation in *F. lycopersici* and in *F. vasinfectum* could be fairly well controlled. Once the suitable conditions were established, the appearance of color was consistent, especially when *meso*-inositol was incorporated in the medium.

Neither of the species had antibacterial activity when tested by the cross-streak agar plate method against the standard Gram positive and Gram negative bacteria.

SUMMARY AND CONCLUSIONS

A comparative study of two species of Fusaria demonstrated that both produce the pigment, lycopersin, which was formed, and remained deposited, in the mycelium. Both species grew on many organic substrates and within a wide range of carbohydrate concentration, temperature, and pH; the maximum formation of pigment, however, appeared within pH range 2.7–4.5. Cu ion in trace amounts stimulated pigment formation in *F. lycopersici;* Zn ion remained without effect in this respect.

Besides C_6-compounds, other molecules having shorter carbon chains participated in the formation of lycopersin. This fact may signify that the C_6 molecule was broken down into smaller units before being resynthesized into the pigment with an aromatic structure. The behavior of *meso*-inositol in this connection was significant. The mechanism of its utilization, however, is not known and a search did not reveal any pertinent information. *meso*-Inositol was the best carbon source for the production of lycopersin by *F. vasinfectum;* in addition, lipides were abundantly formed. When ultraviolet absorption spectra were determined on the free oils, the presence of sterols was demonstrated, whereas the isomerized oils revealed the typical absorption spectra of straight-chain unsaturated fatty acids. It follows that *F. vasinfectum* utilized *meso*-inositol for both the synthesis of compounds with ring structure (steroids, pigment) and straight-chain fatty acids. In order to achieve the synthesis of the latter, the ring must be split. When these observations are considered as a whole, it may be concluded that *meso*-inositol is converted by Fusaria into some simpler unit before the synthesis of the diverse products can take place.

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EFFECT OF COBALT ON PRODUCTION OF STREPTOMYCIN AND VITAMIN B-12 BY STREPTOMYCES GRISEUS[1]

P. A. Principe[2] and H. H. Thornberry

The availability of a chemically defined medium (13, 14) for *Streptomyces griseus* (Krainsky) Waksman & Henrici (15) permits further studies on the metabolism of this organism under controlled conditions of nutrition. Studies on the effect of cobalt on *S. griseus* were undertaken to determine whether this microelement influenced the metabolism of this organism relative to the production of streptomycin and vitamin B-12. This vitamin having growth-promoting activity for *Lactobacillus lactis* Dorner (12) was originally isolated from liver (9) and subsequently obtained from the culture broth of several microorganisms, including a grisein-producing strain of *S. griseus* (10). Increased production of vitamin B-12 has been reported when *S. griseus* is grown on the "N-Z Amine" medium supplemented with cobalt (4). The studies herein involve the measurement of four responses (growth, pH, streptomycin production, and vitamin B-12 synthesis) by *S. griseus* when grown in submerged cultures on the basal synthetic medium (13, 14) supplemented with varied amounts of cobalt. The purpose of this paper is to report the results obtained.

MATERIALS AND METHODS.—The basal synthetic medium used was a modification[3] of the chemically defined medium proposed by Thornberry and Anderson (14) for submerged culture. In the preparation of the complete medium for each of the several concentrations of cobalt, the amount of cobalt (as aqueous solution of $CoCl_2.6H_2O$) necessary for the desired final concentration in 300 ml. was added to 150 ml. of double-strength basal medium previously adjusted with NaOH or HCl to a pH of 7.2. Distilled water was added to make the volume up to 300 ml. and this was placed, in 100-ml. aliquots, in fermentation containers, square 1-qt. Mason jars fitted with screw-cap tops consisting of metal rims with a cotton-gauze filling for aseptic aeration. The containers were subsequently autoclaved at 121° C. for 20 min.

The culture of *Streptomyces griseus* used was a subculture of Waksman's strain No. 9 from No. 18–16 originally obtained from heavily manured field soil (11).

Streptomycin (A and B not distinguished) was estimated as micrograms of the base per ml. in duplicate assays, by the paper-disc method against *Bacillus subtilis* cultured at 30° C. (6).

H-ion concentrations were determined with a glass electrode Beckman pH meter.

Growth of *S. griseus* was estimated qualitatively on an arbitrary scale of 0 to 10 by visual comparison with that on a corn steep medium[4] rated as 10 or "excellent."

Growth conditions were controlled in an air-conditioned culture room at 28° C. (± 1°) and relative humidity 50 per cent (± 5 per cent). Submerged cultures were maintained on a shaking machine imparting a rotary motion of 220 r.p.m. The cultures were grown for 5 days and samples for pH, growth, and streptomycin determinations were removed aseptically on the third, fourth, and fifth days of fermentation. The remaining culture medium on the fifth day was frozen and stored for the vitamin B-12 assays.

Vitamin B-12 was assayed (see acknowledgment) by the plate *Lactobacillus leichmannii* method (1). Each determination was made on a combined sample from three fermentation jars after 5 days of fermentation.

The inoculum consisted of 2 ml. per jar of a 48-hr. shake-flask culture of *S. griseus* on corn steep medium which previously had been inoculated with growth (2 weeks) from nutrient agar slants obtained by transferring the organism from a soil culture stored at 4° C.

The effect of cobalt on growth of the test organism in the assay plates was determined by applying aqueous solutions of the various concentrations of cobalt directly to the assay discs on the medium and then incubating the plates as in the usual assay. Zones of inhibition were measured.

The effect of cobalt on the assay of streptomycin was determined by adding a known amount of streptomycin (25 μg. per ml. final volume) to the non-inoculated synthetic medium with varied concentrations of cobalt, and then assaying the mixture in the usual manner. Also, the same concentrations of cobalt in aqueous solutions were mixed with streptomycin and assayed.

EXPERIMENTS AND RESULTS.—As a means of spotting the effective region of cobalt concentration in the medium, a preliminary experiment was carried out using a wide range of cobaltous chloride concentration (intervals of 1 and 5×10^{-8} to 1×10^{-1} molal). In each of triplicate runs each concentration of cobalt was triplicated, making nine samples for each variable. The results (means from 18 assays) indicate that increased streptomycin production occurred only at 1 and 5×10^{-3} molal. For the 3-, 4-, and 5-day fer-

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[3]A 0.1 molar concentration of cerelose was used to avoid the delay in the production of an alkaline medium when higher concentrations of sugar are present.

[4]Corn steep liquor, 5 gm.; cerelose, 10 gm.; peptone, 5 gm.; and sodium chloride, 5 gm. per liter of distilled water; adjusted to pH 7.2.

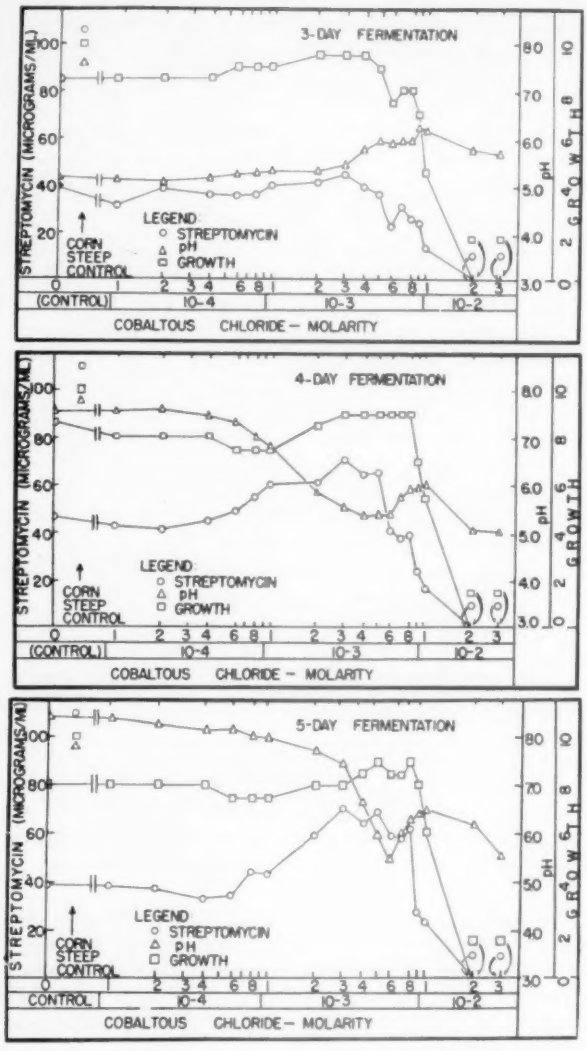

FIG. 1. The effect of concentration of cobalt in a chemically defined medium on three responses (streptomycin production, pH, and growth) of *Streptomyces griseus* in submerged culture: top, 3 days; middle, 4 days; bottom, 5 days.

mentations, the basal medium without cobalt yielded 15, 28, and 30 μg./ml. respectively, whereas 0.001 M. cobalt gave 25, 36, 28 μg./ml. and 0.005 M. gave 26, 48, and 62 μg./ml. At the cobalt concentrations giving increased production of streptomycin, growth of the organism was stimulated and the pH of the medium remained lower than at the other concentrations.

From the guiding information supplied by the preliminary experiment, the effect of cobalt on *S. griseus* was determined at close intervals of concentration near the effective region mentioned above. The procedure followed was identical to that in the preliminary experiment. The results (means from twelve assays on six replications in two experiments) coincide with those of the preliminary experiment (Fig. 1).

In the experiment to determine any effect of cobalt on the assay of streptomycin, there was no influence except for the 21 per cent reduction in streptomycin inhibition at 0.1 molar cobalt and 46 per cent reduction at 0.5 molal. There was no influence on streptomycin assay by the basal medium without cobalt or with cobalt through the range of concentration tested, 1×10^{-4} to 3×10^{-2} M.

In the experiments to detect the effect of cobalt on the test organism, cobalt in aqueous solution was without effect except at 0.5 M. which accounted for a 20-mm. zone of inhibition of the test organism. In the presence of 25 μg. of streptomycin/ml., the zone of inhibition of this concentration of cobalt was 15 mm., equivalent to 14 μg./ml. of streptomycin. Thus the combination of 0.5 M. cobalt with streptomycin resulted in a zone of inhibition less than that caused by either streptomycin or cobalt when alone. Cobalt in the basal medium was without effect on the test organism at the highest concentration tested (0.03 M.). Basal medium without cobalt and with cobalt up to 8×10^{-3} M. when agitated and aerated gave a trace of inhibition. None of the higher concentrations of cobalt tested (up to 0.03 M.) gave detectable inhibition.

In the experiment to detect toxic substances that might arise from cobalt catalysis in the noninoculated medium during aeration and agitation, the assay plates showed faint inhibition rings at the edges of the pads from the basal medium without cobalt and with cobalt up to 0.008 molal. However, at 0.009 to 0.03 molar cobalt in agitated medium, no inhibition was detected. No inhibition occurred from the nonagitated and nonaerated medium with or without cobalt. These data suggest that agitation and aeration of the medium without the organism causes the production of some substance that is slightly toxic to the test organism. They also indicate that cobalt is not involved in the catalysis. Cobalt at the higher concentrations even interferes with the catalysis.

In a study of the morphological changes in *S. griseus*, the general growth cycle was similar to that reported by Carvajal (2), who used *S. griseus* in a synthetic medium in submerged cultures. Good sporulation or fragmentation preceded the peak in streptomycin production. In those concentrations of cobalt considered favorable for streptomycin production, moderate to heavy production of spores along with good mycelial growth and mycelial fragments occurred in the third day of fermentation. The 4-day cultures showed an increase in mycelial growth and fewer spores as contrasted to the 5-day cultures, which showed a significant predominance of spores in long chains with few mycelial fragments. Also, it was noted that in the higher concentrations (10^{-2} M.), at which growth was poor and slow and streptomycin production was low, spore formation was sparse in proportion to the amount of mycelial fragments.

It was noted in these and earlier experiments that the initial pH of the corn steep medium appeared to

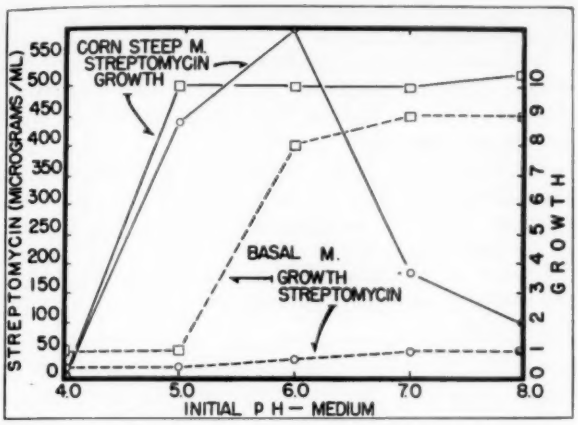

FIG. 2. The effect of initial hydrogen-ion concentration of the basal medium and of the corn steep control medium upon growth and streptomycin production by *Streptomyces griseus* in submerged culture for 3 days.

affect the production of streptomycin. Results (Fig. 2) from the experiment designed to measure such responses indicate that decidedly more streptomycin (600 μg./ml.) is produced in 3 days when the medium is at pH 6.0 at the start of the fermentation than when the fermentation is started at pH 7.0 (188 μg./ml.) or at pH 5.0 (440 μg./ml.). Growth was essentially not affected by the initial reaction from pH 5.0 to 8.0. The best initial pH for growth and streptomycin production in the basal medium was pH 7.0 and 8.0.

The results of the vitamin B-12 assays (Table 1) are presented for their value as records. Although they include an insufficient number of samples for a conclusion, they do suggest that the concentration of cobalt affects the synthesis of vitamin B-12. Basal and corn steep media without added cobalt that were not inoculated had no detectable vitamin B-12.

DISCUSSION.—The production of streptomycin is noticeably increased by the presence of cobalt between 0.002 and 0.008 molar concentration of cobaltous chloride. These increases in yield (55, 83, 68, 81, 56, 55, and 63 per cent respectively for the concentrations of cobalt) are more noticeable when they are considered as percentages of increase over the control. Although the yield of streptomycin is not high from this medium, the chemically defined medium provides a controlled environment by which the effect of cobalt on the organism may be identified.

TABLE 1.—*Amount of vitamin B-12 in 5-day fermentation of synthetic medium containing varied concentrations of cobaltous chloride (assays by the plate L. leichmannii method in mgm./ml.)*

$CoCl_2$ molarity	B–12 (μg/ml.)	$CoCl_2$ molarity	B–12 (μg/ml.)
None[a]	0.16, 0.36	1×10^{-3}	0.19, 0.24
1×10^{-8}	0.05	2×10^{-3}	0.30
1×10^{-7}	0.33	3×10^{-3}	0.24
1×10^{-6}	0.31	4×10^{-3}	0.27
1×10^{-5}	0.08	5×10^{-3}	0.29
1×10^{-4}	0.38, 0.37	6×10^{-3}	0.15
2×10^{-4}	0.40	7×10^{-3}	0.10
4×10^{-4}	0.39	8×10^{-3}	0.24
6×10^{-4}	0.21	9×10^{-3}	0.08
8×10^{-4}	0.29	1×10^{-2}	0.08
		None—corn steep[a]	0.11, 0.51

[a]Media not fermented contained no assayable B–12.

Growth of the organism is only slightly influenced by cobalt except for retardation or inhibition at 0.009 M. and greater concentrations (Fig. 1). These observations suggest that cobalt is not an important essential minor element for the growth of the organism.

Hydrogen-ion concentration of the medium on the third day of fermentation decreases as the concentration of cobalt is increased (Fig. 1, top). However, on the fourth day (Fig. 1, center) and the fifth day (Fig. 1, bottom), there is an increase in acidity at some concentrations, and a lesser increase in acidity at other concentrations of cobalt stimulating streptomycin production. Below these amounts of cobalt, the medium became alkaline as is the case with most media. These responses indicate that cobalt at suitable concentrations stimulates certain channels of metabolism of *S. griseus*. The mechanism may be related to sugar utilization, since an increase in the concentration of glucose in the basal medium without cobalt results in an initial decrease in alkalinity in the medium (14). Or, the mechanism might be related to relatively sluggish or retarded reactions involved in the utilization of organic acids produced. Some organic acids when substituted for lactic acid in this medium are utilized by the organism. However, only lactic acid causes any appreciable increase in the production of streptomycin (5). If a more favorable medium supplying nutrients for balanced mechanism were provided, optimal concentration of cobalt or high glucose concentration might not result in delayed alkalinity in the medium. The results herein refute any claim that streptomycin production is related to the alkalinity of the medium.

The specific role of cobalt in the metabolism of this organism is not apparent from the results of these studies. However, by virtue of its ability to affect growth, stimulate the production of streptomycin, and cause acidity to develop in the medium, cobalt may be classified as Porter's (8) "nutritive trace element" for *S. griseus*. Although these responses attributed to cobalt may be related to the other minor elements and their balance in the medium, the influence is not totally dependent upon these additional elements since cobalt without added Fe, Zn, Mn, and Cu stimulates streptomycin production in this medium (14). Other cases of minor elements influencing the production of antibiotics (7) and the activity of enzymes produced by microorganisms (3) are known. Cobalt may not necessarily act in a manner that is directly stimulative. It may act in the capacity of creating new pathways of metabolism or blocking certain pathways, and may thereby force or provide additional courses of metabolic progression.

An explanation for the effect of initial pH of the

medium on streptomycin production is not apparent from the results of these studies. It is reasonable to expect that the response may be relative to the production of nutritive metabolites at the initial pH values. In addition, the response may be related to the availability of the minor elements by virtue of the solubility of their salts in an acid medium. The failure of the synthetic medium to behave similarly may be due to the inadequacy of this medium to support the metabolism achieved in the favorable corn steep medium. The response from media having varied initial pH values indicates that more attention should be given to this factor in experimental and commercial production of streptomycin.

SUMMARY

Production of streptomycin by *Streptomyces griseus* is increased by cobaltous chloride at concentrations of 0.002 to 0.008 M. in the synthetic medium.

The amount of fungus growth is not materially influenced by the concentration of cobaltous chloride until inhibitive concentrations of 0.009 M. or greater are available.

Hydrogen-ion concentration of the medium is reduced during fermentation except at the concentrations of cobaltous chloride that stimulated the production of streptomycin.

The initial pH of the medium has a marked effect upon growth and streptomycin production in the corn steep medium, but has a marked effect only on growth in the synthetic medium.

Production of vitamin B-12 appears to be affected by the concentration of cobaltous chloride in the synthetic medium.

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STUDIES ON SYSTEMIC FUNGICIDES. II. BEHAVIOR OF GROUPS OF REPORTED CHEMOTHERAPEUTANTS [1]

S. H. Crowdy [2] and Mary-Elizabeth Davies [3]

The treatment of plant disease with chemicals which operate within the host plant, usually referred to as chemotherapy, is a relatively recent development in plant pathology. However, sufficient evidence has been accumulated to establish the method as one of considerable promise.

In general it can be said that the symptoms of plant diseases result either directly, from the activity of the pathogen, or indirectly, from the host's reaction to toxins which the pathogen produces. In either case the final expression of symptoms is the result of the balance between the action of the pathogen and the reaction of the host. Thus the effect of the disease may be reduced by increasing the natural resistance of the host, by reducing the growth of the pathogen, or by antidoting the toxins which it produces. The main problem has been approached from all three aspects by different investigators, using different methods of assessment and usually different types of compounds on different diseases. In this work an attempt has been made to assess the effect, on a typical leaf spot, of some of the chemotherapeutants reported to be effective in reducing vascular diseases.

A difficulty inherent in this type of work is a general vagueness in the concepts involved. This is unavoidable at the present stage because of the complexity of the biological system under investigation. Some of the assumptions made are as yet without experimental support; the only fact proven beyond reasonable doubt is that the application of certain chemicals to the plant, externally or by injection, will reduce disease, often at a considerable distance from the point of application. The simplest way to explain this effect would be to assume that the chemical is taken up by the plant and is transported, either unchanged or as a degradation product, to the site of disease. At the disease site the method of action is again problematical, and where one has been suggested it has been deduced from indirect evidence. Caroselli and Feldman (1) assume that Carolate, a formulation which will in certain circumstances control Dutch elm disease, acts by neutralizing the toxin rather than by checking the pathogen. They base this assumption on *in vitro* observations on toxin formation and the performance of the formulation as a fungicide. A similar type of argument led Crowdy and Wain (2) to attribute the effect of certain of the aryloxyaliphatic acids on chocolate spot of bean to systemic fungicidal action. Within these limitations, the compounds described below will be defined as acting as systemic fungicides when the evidence suggests that they are freely distributed within the host and act directly on the growth of the pathogen, and as toxin inactivators when they are believed to neutralize the toxic products of the pathogen rather than check its development.

EXPERIMENTAL METHODS.—The results reported are, for the most part, concerned with testing the activity of compounds as systemic fungicides which have already been reported as effective in the control of wilt diseases. Some of these compounds may act against wilt diseases as systemic fungicides; others are believed to act as toxin inactivators. The methods employed have already been described in some detail (2) and will be sketched briefly here. Plants of the host chosen, broad bean (*Vicia faba*), were raised from seed in pots in soil until they reached a convenient size, when they were dug up and the roots were washed in water. After 7 or 8 days in an aqueous solution of the compound under test, in 1-pt. Mason jars, the plants were sprayed to run-off with spores of *Botrytis fabae* Sadinia suspended in 1 per cent dextrose solution, and then held in a saturated atmosphere for 3 days. Typical leaf spots developed during this time. Effective treatment reduced the size of individual lesions rather than their number, and the effect was assessed on three (occasionally four) plants per treatment by measuring the diameters of 10 lesions on each of three or four leaves per plant. Because age of the leaf had a considerable effect on size of the lesion, care was taken to compare corresponding leaves on different plants. This method of assessment provided a means of measuring the activity on the leaves of a compound which had been applied to the roots.

No attempt was made to standardize the inoculation procedure, and no precise control was maintained over actual conditions. It was, therefore, impossible to compare the results of one trial with another. However, all the plants in one trial were sprayed to run-off with the same spore suspension, providing uniform inoculum within each trial. Also, during the trial the plants were subject to the same external conditions. Further, the assessment method of measuring mean lesion diameter avoided any minor variations in the inoculation technique. The intensity of the disease visible to the eye depends on leaf area damaged rather than on diameter of individual lesions and, as already explained (2), the area damaged is directly related to the square of the diameter. For this reason the results are presented as percentage areas damaged, calculated from the square of the

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means of the lesion diameters, and the treatment effects are compared as percentages of the controls. This standardization allows a general comparison between trials, but comparative figures must be treated with considerable caution.

Observations were made also on the fungicidal activity of the compounds themselves by means of standard spore germination tests using *Alternaria solani* as the test organism. The criterion for fungitoxicity chosen was the LD 50 estimated from the conventional probit transformation of the dosage mortality curve. The actual activity of the compounds under investigation as systemic fungicides will depend as much on their concentration at the point of action as on their intrinsic fungicidal activity. Their effectiveness will, therefore, depend also on their phytotoxicity, the readiness with which they are absorbed by roots and translocated to leaves, and the extent to which they accumulate within leaves.

The compounds tested were received from a variety of sources. The hydroxyquinoline derivatives were supplied by the Connecticut Agricultural Experiment Station, the diazo compounds (D4, S3, S5) by Industrial Dyestuffs Company, the thioureas, p-aminobenzoic acid, p-aminobenzene sulfonamide, Pontamine Sky Blue 6BX (5R1131) and Erie Fast Orange C.G. (5R1196) by Merck and Company, and the complex metallic dinicotine salicylates by the Eastern Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, U. S. Department of Agriculture.

COMPOUNDS UNDER TEST.—*Hydroxyquinoline derivatives.*—Certain derivatives of hydroxyquinoline are known as fungicides. Some have been selected as possible chemotherapeutants in the treatment of Dutch elm disease because of their ability to check the growth of *Ceratostomella ulmi*. Within this group two compounds were effective (10): 8-hydroxyquinoline benzoate and 8-hydroxyquinoline sulfate. The latter also neutralized the toxin of *C. ulmi*. The effect of the 8-hydroxyquinoline benzoate appeared to be more lasting (10), and in further trials this compound was effective against Dutch elm disease in the field (6). Both compounds also will reduce Verticillium wilt of eggplant (9), and 8-hydroxyquinoline salicylate also is recorded as reducing systemic infections of a blight of red kidney bean caused by *Xanthomonas phaseoli* (5). The effect of these compounds in reducing chocolate spot of broad bean is summarized in table 1. All the quinolines tested were toxic to the bean at the concentration of 10 p.p.m., causing wilting and root damage; 2-hydroxy,4-methylquinoline induced a mild leaf necrosis. They could be used safely at a concentration of 1 p.p.m.

It would appear from table 1 that a number of these compounds can be taken up by the host and translocated, though the reduction of disease due to 8- and 2-hydroxyquinoline was of doubtful significance. These findings confirm the observations of Zentmyer, Horsfall, and Wallace (10), who found that

TABLE 1.—*The effect of hydroxyquinoline derivatives on chocolate spot of broad bean, when applied to the roots at a concentration of 1 p.p.m., and their toxicity to spores of Alternaria solani*

Compound	Reduction in disease percentage	LD 50 (p.p.m.)
8-Hydroxyquinoline	15*	3
2-Hydroxyquinoline	12	75
8-Hydroxyquinoline sulfate	31**	1
8-Hydroxyquinoline benzoate	35**	1
8-Hydroxyquinoline succinate	28**	1
8-Hydroxyquinoline,5-sulfuric acid	28**	220
2-Hydroxy,4-methylquinoline	31**	380

*Significant: P = 0.05.
**Significant: P = 0.01.

8-hydroxyquinoline sulfate and 8-hydroxyquinoline benzoate were markedly more effective than 8-hydroxyquinoline. However, it will be noted (Table 1) that the ability of compounds to increase the resistance of broad bean to *Botrytis* is not necessarily correlated with ability to inhibit spore germination of *Alternaria solani*.

Azo dyes.—The dye Helione orange, which is presumed to contain diaminoazobenzene dihydrochloride as the active principle, has been reported by Howard and Caroselli (8) as inhibiting the growth of *Phytophthora cactorum*, the cause of bleeding canker of maple and certain other hardwoods, and inactivating the toxin which the pathogen produces. They report some success when using this product in the treatment of the disease of maple (8). Howard and Caroselli believe that the therapeutic effect is due to the toxin-antidoting properties of the product. Zentmyer, Horsfall, and Wallace (10) also observed that this compound neutralized the toxin of *C. ulmi*, but was ineffective in reducing the symptoms of Dutch elm disease.

In view of this six dyestuffs, Helione A, diazo compounds D4, S3, and S5, Pontamine Sky Blue 6BX, and Erie Fast Orange C.G., were used in tests on broad bean. Helione A, presumed to be 2,4-diamino-5-methyl azobenzene hydrochloride, was the only one which consistently reduced the size of lesions and was one of the least phytotoxic compounds examined. In two tests it was used at concentrations of 10, 50, and 100 p.p.m. without damage to the host. There was no significant difference between the treatment effects in either of the tests (Table 2). However, the reduction in disease in the 50-p.p.m. concentration in test 2 just fails to differ significantly from the control. The spore germination tests with the six dyes showed that LD 50 for Helione A was 30 p.p.m.; of the ineffective compounds, one had an LD 50 of 250 p.p.m. and the remainder failed to reduce germination at 500 p.p.m.

Salicylic acid.—Dimond and Stoddard (4, 5) found that salicylic acid reduced the systemic infection of the blight of red kidney bean to about 14 per cent

TABLE 2.—*The effect of Helione A on chocolate spot of broad bean when applied to the roots*

Concentration (p.p.m.)	Reduction in disease percentage	
	Test 1	Test 2
10	31**	22**
50	41**	11
100		22**

**Significant: P = 0.01.

when applied to the roots, though it appeared to be phytotoxic at high temperatures. Their evidence suggests that the compound is readily mobile but is rapidly metabolized. The compound also was tested by Feldman, Caroselli, and Howard (3) against Dutch elm disease. When applied alone to the soil by subsurface impregnation it had little effect, but gave good control when formulated with lime. When tested with the broad bean the compound was slightly injurious to the roots at 10 p.p.m. At this concentration the reduction in disease percentage was 28; at 1 p.p.m., 39 per cent. In both cases the reduction was statistically significant at a probability (P) of 1 per cent. This compound is known as a fungicide yet in the present trials had an LD 50 near 500 p.p.m.

Complex metallic dinicotine salicylates.—Data in this report suggest that salicylic acid can be translocated within the plant. It is, therefore, of interest to observe the behavior of a series of complex metallic dinicotine salicylates (Table 3), particularly since some members of the series have proved to be good fungicides. In both tests the compounds significantly reduced disease, though there is a considerable difference in their performance in the two trials. This variability between tests is a common experience in this work and is probably unavoidable without very strict control of environmental factors. However, within each test the relative performance of the compounds is about the same. Cadmium, cobalt, and manganese caused some root necrosis and slight leaf damage at 10 p.p.m. in the first trial. In the nickel treatment, roots were severely damaged. The anomalous behavior of the zinc salt probably also reflects its phytotoxicity. For actual comparison between the performance of these compounds at 1 and 10 p.p.m., the data in the table are misleading, since disease reduction is greater at the higher concentration on young leaves, but this advantage is lost on older leaves (Fig. 1). This interaction of leaf age on treatment is highly significant and may be caused by a premature senescence of leaves in the plants treated with the higher concentration, reflecting the damage due to treatment.

TABLE 3.—*The effect of metallic dinicotine salicylates on chocolate spot of broad bean when applied to the roots, and their toxicity to the spores of Alternaria solani*

Metallic radical and concentration (p.p.m.)	Reduction in disease percentage		LD 50 (p.p.m.)
	Test 1	Test 2	
Cadmium, 10	61**	29**	8
Cadmium, 1	....	15	....
Cobalt, 10	60**	....	75
Copper, 10	....	18	8
Copper, 1	....	21*	....
Manganese, 10	76**	21*	....[a]
Manganese, 1	....	34**	....
Nickel, 10	41**	....	110
Zinc, 10	—21*	—7	180

*Significant: P = 0.05.
**Significant: P = 0.01.
[a]No inhibition at 500 p.p.m.

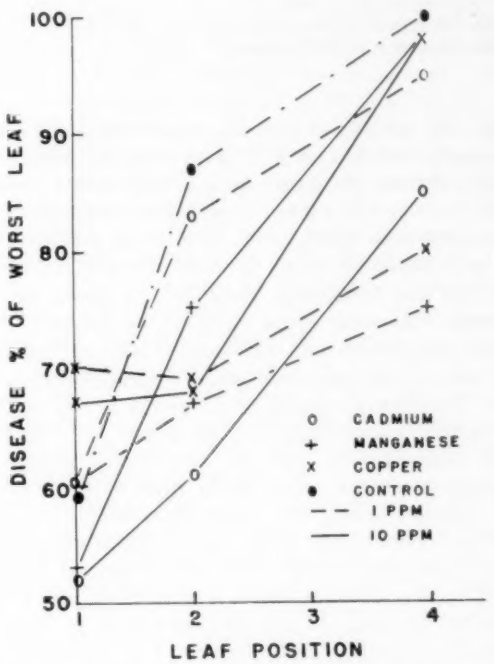

FIG. 1. The effect of metallic dinicotine salicylates on chocolate-spot infections on leaves of different ages. No. 1 was the youngest leaf assessed.

Para-aminobenzoic acid and para-aminobenzene sulfonamide.—Zentmyer, Horsfall, and Wallace (10) found that para-aminobenzoic acid arrested wilt to some extent in Dutch elm disease, though the effect was not striking. Para-aminobenzene sulfonamide was too phytotoxic for inclusion in field tests (10).

Against chocolate spot of bean, p-aminobenzoic acid was among the more active compounds tested; it reduced disease 37 per cent at 1 p.p.m. and 73 per cent at 10 p.p.m. The last figure is probably not typical of the compound, since in the same trial 2,4,6-tricholorophenoxyacetic acid caused a reduction of 78 per cent, which was greater than that usually recorded. It would appear, however, that this compound will reduce disease to about the same extent as 2,4,6-trichlorophenoxyacetic acid. Para-aminobenzene sulfonamide had no effect. These results also reflect the *in vitro*

TABLE 4.—*The effect of ureas and thioureas on chocolate spot of bean when applied to the roots, and their toxicity to spores of Alternaria solani*

Compound (concentration, 1 p.p.m.)	Reduction in disease percentage	LD 50 (p.p.m.)
Urea (at 10 p.p.m.)	22*	....[a]
4-Chlorophenyl urea	2	1000
N N'bis (2,4-dichlorophenyl) urea	31**	....[b]
4-Chlorophenylthiourea	43**	35
N N' (4-chlorophenyl) thiourea	23**	480
N N'bis (b-naphthyl) thiourea	51**	....[b]

*Significant: P = 0.05.
**Significant: P = 0.01.
[a]No inhibition at 10 p.p.m.
[b]No inhibition at 1000 p.p.m.

fungicidal activity of the two compounds, since p-aminobenzoic acid has an LD 50 of 34 p.p.m. against *A. solani*, whereas the figure for p-aminobenzene sulfonamide is about 210 p.p.m. It is unwise to draw conclusions from this effect, since there is no evidence that the sulfonamide is taken up or translocated.

Ureas and thioureas.—Horsfall and Zentmyer (7) reported that urea would antidote the toxins of both Dutch elm disease and Verticillium wilt on eggplant, and that it also exercised some chemotherapeutic effect. Feldman, Caroselli, and Howard (3) found that by itself this compound had no effect, but that lime, urea, salicylic acid, and azo dye combined gave an effective treatment. This is the basis of the formulation Carolate which was reported as giving considerable protection in the treatment of Dutch elm disease (1). There is no claim that this compound has any effect on the pathogen and it is considered purely as a toxin inactivator. In a preliminary trial on elms by Zentmyer, Horsfall, and Wallace (10) thiourea was severely damaging.

The results of trials with these compounds on chocolate spot of bean are in table 4. At 10 p.p.m. all compounds except urea severely damaged bean roots. 4-Chlorophenylurea also caused severe interveinal necrosis, which suggested that it had been translocated. The results of the test using a more dilute solution confirm this supposition.

DISCUSSION.—Concepts in plant chemotherapy are still too vaguely defined to permit definite conclusions as to the type of compound which is likely to be active and the way in which it acts. Most of the chemicals tested are not necessarily specific to a particular type of disease, since some of those selected for effectiveness against a variety of wilt diseases will also reduce the spread of a leaf spot. In two of the groups examined, the hydroxyquinolines and the azo dyes, the same compounds might be selected as effective whether tested against wilt or leaf disease. Where comparative tests have been made the only compound which appears to approach 2,4,6-trichlorophenoxyacetic acid in activity is p-aminobenzoic acid. Even though the effect of the remainder is relatively small, the tests illustrate that a variety of compounds may be expected to act internally on disease in plants.

Observations on the fungitoxicity of these compounds support the hypothesis that some may be acting as systemic fungicides, and the minor discrepancies between the results of the *in vitro* and *in vivo* tests can be attributed to the ability of the host to translocate and accumulate the different compounds. This explanation might well be applied to the results of the tests with the hydroxyquinoline derivatives. The behavior of the ureas and thioureas and of manganese dinicotine salicylate dihydrate requires a different explanation and at present there is little evidence to suggest a likely alternative. It is probably significant that in these cases the fungitoxic and nonfungitoxic members of the group produce effects of essentially the same order of magnitude, suggesting a similar mode of action.

SUMMARY

Several groups of compounds reported as chemotherapeutants were tested for their ability to reduce the intensity of chocolate-spot disease of broad bean. In general, the compounds which had been reported as effective against other diseases effected some reduction in leaf-spot percentage.

With the azo dyes and the hydroxyquinolines there was also some correspondence between the behavior of individual members of the groups in their effect on different diseases.

In most cases there was no close relationship between the activity of the compounds in spore germination tests and their behavior on broad bean. However, only the azo dye with fungicidal potency was effective.

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PATHOGENICITY OF SOME PLANT-PARASITIC NEMATODES FROM FLORIDA SOILS. I. THE EFFECT OF DOLICHODORUS HETEROCEPHALUS COBB ON CELERY[1]

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Dolichodorus heterocephalus Cobb was first found in fresh water at Douglas Lake, Michigan, and Silver Spring, Florida.[3] At the time of its original description, this species was regarded as an aquatic, free-living form and no mention was made of its association with plants. Taylor,[4] in a survey of plant-parasitic nematodes in Florida, described nematodes of a genus closely related to *Paratylenchus* which were associated with a disease of celery known locally as "red root." He stated that the exact relation of the *Paratylenchus* sp. to "red root" could not be ascertained and that one field of celery (*Apium graveolens* L.) was observed in which 50 per cent of the plants were stunted by this disease. Steiner[5] later identified as *Dolichodorus heterocephalus* the nematodes observed by Taylor, and stated that they feed on roots, which they puncture from the surface with their long needle- or awl-shaped buccal stylets.

Dolichodorus heterocephalus frequently has been found around the roots of some Florida crop plants,[6] but since experimental evidence of its pathogenicity was lacking, the following experiment was conducted.

MATERIALS AND METHODS.[7]—Seven-week-old celery seedlings were transplanted to 5-in. pots containing a sterilized mixture of 2 parts sand and 1 part soil. A total of 30 plants was divided into three lots of 10 plants each. Inoculum for each plant was pipetted from a stirred water suspension of nematodes. This suspension contained 77 per cent D. heterocephalus, 10 per cent *Hoplolaimus coronatus* Cobb, 9 per cent saprophytic species of the genera *Rhabditis*, *Eucephalobus*, *Acrobeles*, *Diplogaster*, and *Acrobeloides*, and 4 per cent other plant-parasitic or suspected plant-parasitic forms of the genera *Belonolaimus*, *Aphelenchoides*, *Hemicycliophora*, *Dorylaimus*, and *Trichodorus*. The volume of suspension pipetted to each plant was regulated so that each plant in the first lot was inoculated with 500 nematodes of the species *D. heterocephalus* and each plant in the second lot received 1000 nematodes of the same species. Plants in the third lot were left uninoculated and used as controls. All plants were randomized and watered immediately following inoculation.

After 52 days at a mean soil temperature of 22° C., plants were harvested and data were obtained of plant weight, root weight, top weight (weight of the above-ground parts), and length of the longest leaf. Nematodes were recovered from the soil in which each plant had been growing by means of the Cobb[8] screening-gravity method. Nematodes retained by 60- and 150-mesh screens were placed in a water suspension, and the numbers in aliquots pipetted from the stirred suspensions were counted. The total recovered from each pot was computed.

RESULTS.—As early as 3 weeks after inoculation, plants which had received 1000 nematodes were noticeably stunted and chlorotic. Chlorosis and necrosis were most evident in the older leaves. Plants which had received an inoculum of 500 nematodes were also slightly stunted and chlorotic, but not so noticeably as those in the 1000-nematode treatments.

Differences between treatments became more pronounced with time until the experiment was terminated (Table 1). Figure 1 shows five representative plants from each treatment at this time. When soil was washed from root systems, infected plants, particularly those that had been inoculated with 1000 nematodes, had an excess of stubby secondary roots and a shortage of fine feeder roots.

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[6]As stated to the senior author in correspondence from J. R. Christie.

[7]The authors are indebted to J. R. Christie and V. G. Perry, Division of Nematology, Bureau of Plant Industry, Soils, and Agricultural Engineering, Central Florida Experiment Station, Sanford, for their indispensable cooperation in supplying the nematode inoculum used in this experiment.

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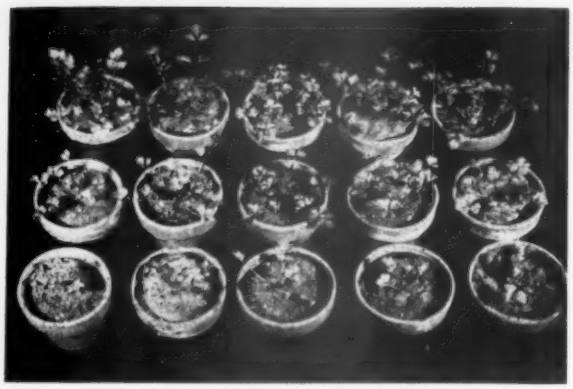

FIG. 1. Celery plants grown in soil infested with *Dolichodorus heterocephalus:* upper row, noninoculated controls; inoculated with (middle row) 500 and (lower row) 1000 nematodes.

Counts of nematodes recovered from soil in which plants had grown indicated that *D. heterocephalus* was the only plant-parasitic species that had increased during the course of the experiment. This species was the only one present in numbers large enough to produce the injury observed. An average of 540 *D. heterocephalus* was recovered from pots to which 500 nematodes had originally been added. Since complete recovery of all nematodes in soil is not possible with the Cobb screening-gravity method and since many larval *D. heterocephalus* were observed among the nematodes recovered, it was evident that this species was reproducing in the pots. An average of only 254 *D. heterocephalus* was recovered from pots which had received an initial inoculum of 1000 nematodes. The authors believe that the root systems of plants receiving 1000 nematodes were reduced so severely that they could not support so many nematodes and many of the nematodes perished.

TABLE 1.—*The effect of two levels of inoculum of Dolichodorus heterocephalus on growth of celery plants*

Treatment	Weight, in grams			Length, in inches (longest leaf)
	Plants	Tops	Roots	
Control	14.47[a]	5.87	8.63	5.74
500 nematodes	8.15	3.76	4.39	4.79
1000 nematodes	4.04	1.81	2.23	3.62
LSD, 5 per cent level	2.78	1.19	1.72	0.59
1 per cent level	3.76	1.61	2.33	0.80

[a]Each value represents the average of 10 replicate plants.

SUMMARY AND CONCLUSIONS

A mixed population of nematodes that contained 77 per cent *Dolichodorus heterocephalus* caused a serious decline of celery seedlings. *D. heterocephalus* was the only species which increased in number while confined to pots of soil in which celery was growing. Therefore, it is assumed that *D. heterocephalus* caused the decline. Symptoms produced experimentally by inoculation included stunting, chlorosis, production of stubby secondary roots, and a reduction in number of fine feeder roots. *D. heterocephalus* was shown to cause celery rootlet injury and thus may be a factor in the "red root" complex in Florida.

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EFFECT OF SPRAYING WITH ZINEB ON THE GROWTH AND ZINC CONTENT OF THE TOMATO PLANT[1]

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Certain organic zinc fungicides, principally zineb (zinc ethylene bisdithiocarbamate) and ziram (zinc dimethyl dithiocarbamate) have been shown (1, 2, 4, 6, 8, 9) to have at times a beneficial effect on yield when applied in the field to such plants as potatoes, tomatoes, cantaloupes, and cucumbers. There has not been unanimity of opinion, however, as to the reason for this phenomenon. Horsfall and Turner (8) suggested that yields of potatoes sprayed with nabam (disodium ethylene bisdithiocarbamate) plus zinc sulfate and lime, a mixture which forms zineb in the spray tank, were larger than yields of Bordeaux-sprayed plants because the organic zinc fungicide had less lime and, therefore, a lower level of phytotoxicity. Hoyman (9) thought that the zinc in zineb and ziram might be a contributing factor to yield increases. Heuberger (6) advanced the hypothesis that the carbon and possibly the sulfur in the zinc and iron dithiocarbamates might be largely responsible.

Increased growth of field-grown tomato plants which had been sprayed with zineb was noted at Urbana in 1946. As a result of this observation, analyses were made of stems and fruit of these plants as well as plants sprayed with certain other organic fungicides, to ascertain relative absorption or accumulation of zinc. The data from these assays prompted further experimentation with zineb under controlled conditions in the laboratory and greenhouse to determine more accurately to what extent tomato plants are able to utilize the zinc in this compound. At the time this research was first undertaken, no information was available as to how much if any residual, inorganic, water-soluble zinc might be present in zineb. Therefore, chemical and biological assays were made of zineb with this in mind. A brief, preliminary report covering these investigations has already been given (5).

MATERIALS AND METHODS.—*Experiments with field-grown tomatoes.*—Plants of the Garden State variety of tomato were planted in nonreplicated rows 6 ft. apart, and sprayed on July 23, August 5 and 19, and September 3 with several organic fungicides. The fungicides and their concentrations were: Parzate (zineb), 2 lb./100 gal. water; Dithane D-14 (nabam) and zinc sulfate, 2 qt., 1 lb./100; zinc 8-quinolinolate, technical grade, 1 lb./100; Zerlate (ziram), 2 lb./100; and manganese ethylene bisdithiocarbamate, technical grade, 1 lb./100. A wetting agent, Triton B 1956, was added to each spray mixture at the rate of 3 oz. to 100 gal. Sprays were applied with a single-nozzle, orchard-type gun (6/64-in. disk-aperture) adjusted to give a 40°–50° cone at 200 lb. pressure and at approximately 200 gal. per acre.

Samples for analysis were collected on October 5. These consisted of two 6-in. stem sections cut at a distance of 15–18 in. from the main stem of each of the five plants in every treatment, and 10 green fruit, weighing about 4 oz. each, taken at random from individual rows. The stem sections were washed in running tap water, scrubbed lightly with a brush, scraped with a knife to remove the epidermis, and dried at 65° C. for 24 hr. The fruit was washed under running tap water and then wiped thoroughly with clean cotton toweling to remove as much spray residue as possible. The stem and blossom ends as well as wounded areas of the fruit were cut off and discarded. Three slices approximately ⅛ in. thick were cut from the center of each fruit and dried in a forced-draft oven at 65° C. for 8 hr.

Three 10-gm. samples of the stems and of the fruit from each spray treatment and the check were analyzed for zinc by the dithizone mixed-color method (11), using visual comparisons with a series of zinc standards.

Experiments with greenhouse-grown tomatoes. — Seed of the Early Baltimore variety of tomato were planted in quartz sand moistened with tap water. When the seedlings were 2–3 in. tall they were removed from the sand and their roots were thoroughly washed in redistilled water; the seedlings were then placed in the appropriate nutrient solution.

The containers for the culture solutions were 1-liter Pyrex beakers wrapped in black paper and fitted with paraffin-coated Masonite covers. Each beaker held six plants which were supported by 1-in. lengths of Pyrex tubing (inside diameter 7 mm.), spaced equidistant in a circle and fitted through the cover, with the stems of the plants held in the tubing with cotton. A Pyrex-glass aeration tube was placed in the center of each cover and allowed to extend to within ¼ in. of the bottom of the beaker. All cultures were aerated continuously from a compressed air line, with the flow so regulated as to prevent splashing of the solution against the covers. The air was filtered through cotton plugs in the glass aeration tubes. Since relatively high light intensities are conducive to the development of zinc-deficiency symptoms (3), supplemental light was provided by suspending four 300-watt Mazda bulbs 3 ft. above the beakers. During cloudy weather the lights were left on continuously and on clear days were turned on only from 5 p.m. to 11 p.m.

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The complete nutrient solution used throughout this investigation was that of Hoagland and Arnon (7), modified by substitution of potassium dibasic phosphate for potassium acid phosphate. This was done to comply with the purification procedure of Stout and Arnon (13), which is a modification of Steinberg's (12) calcium carbonate method. The final concentrations of the various components in the complete nutrient solution, in p.p.m., were: N, 210; P, 31; K, 273; S, 65; Mg, 24.3; Fe, 1; Mn, 0.5; Zn, 0.05; Mo, 0.05; and Cu, 0.02. When a minus-zinc solution was desired, it was necessary only to omit the zinc sulfate. The hydrogen-ion concentration of the complete solution was pH 5.5. The solutions in the beakers were changed completely every third day with redistilled water added daily to maintain a near-constant level.

Unless otherwise indicated, the spray treatments used throughout this phase of the investigation were a suspension of technical zineb (Dithane Z-78, 98 per cent active) in redistilled water at a concentration of 377 p.p.m.[3] of zinc, and a solution of zinc sulfate ($ZnSO_4 \cdot 7H_2O$, Baker and Adams reagent grade) in redistilled water containing 136 p.p.m.[4] of zinc. The plants were sprayed once, 14 days after they were placed in the culture solution. The sole exception was those plants in one series which were sprayed only after severe zinc-deficiency symptoms were visible. A DeVilbiss No. 152 atomizer was coupled to a compressed air line and to a flask containing the spray material. The plants were sprayed until run-off began, which required approximately 40 cc. for each set of six plants.

The plants were removed from the nutrient solutions at the end of 40 days. Total weight of each six-plant replicate was recorded after the plants had been dried in a forced-draft oven at 65° C. for 24 hr. In one experiment the growing point and the uppermost expanded leaflet of each plant were removed for zinc analysis. These were undifferentiated at the time of spraying and thus were not exposed to zinc sprays to any appreciable extent. The dithizone mixed-color method was used in analysis. Light-transmission readings of the sample solutions were made with a Lumetron photolometer fitted with a 650-mu filter.

Three series of spraying experiments were conducted. A suspension of zineb and a solution of zinc sulfate were sprayed, in series 1, on plants growing in a zinc-deficient nutrient solution but not showing deficiency symptoms; in series 2, on plants likewise growing in a zinc-deficient solution but having severe deficiency symptoms; and in series 3, on plants growing in a complete nutrient solution and not showing deficiency symptoms. There were four treatments in each of the three series. Two treatments consisted of spraying the plants with zineb and with zinc sulfate; the other two were unsprayed controls. The plants in one control were growing in a complete solution and those in the other control in a zinc-deficient solution. Three replicates were employed, each consisting of six plants, making a total of 18 plants per treatment.

Chemical and biological assay of technical zineb.—Ten gm. of technical zineb were shaken for 1 min. in 300 cc. of redistilled water and then separated from the water by filtration through two thicknesses of acid-washed, Whatman No. 50 paper. The zineb was immediately washed off the filter paper and again suspended in redistilled water. After each series of five suspensions and filtrations, the filtrate was analyzed for zinc using the dithizone mixed-color method and a photolometer. Fifty such suspensions and filtrations were carried out.

A suspension of zineb which had been subjected to 30 consecutive washings and one of unwashed zineb, each at a concentration of 377 p.p.m. of zinc, were sprayed on two different sets of tomato plants growing in a zinc-deficient nutrient solution. A set consisted of three replicates of six plants each.

In another test unwashed technical zineb was compared in the same fashion with zineb prepared in the laboratory. In order to eliminate any inorganic, soluble zinc salt, the laboratory-made zineb was prepared by using nabam in excess of that required for complete interaction with the zinc sulfate. The reaction product was filtered and washed repeatedly with redistilled water and after the last filtration was dried for use. Both kinds of zineb were suspended for spraying at concentrations of 377 p.p.m. of zinc.

RESULTS.—*Analyses of field-grown tomato stems and fruits.*—The amount of zinc found in the various stem samples, expressed in p.p.m., was: zineb, 2.5; nabam and zinc sulfate, 1.2; zinc 8-quinolinolate, 1.2; ziram, 1.0; and unsprayed check, 0.9. The zinc content of the fruit sprayed with nabam and zinc sulfate was 0.4 p.p.m.; with ziram and zinc 8-quinolinolate, 0.2 p.p.m.; and with zineb, 0.1 p.p.m. The unsprayed check likewise had 0.1 p.p.m. of zinc.

Response of greenhouse-grown tomato plants to zineb.—The plants sprayed with zineb or zinc sulfate in series 1 were significantly larger than unsprayed plants, but there was no significant difference between

TABLE 1.—*Mean dry weight and zinc content of tomato plants sprayed with zineb and zinc sulfate 14 days after being placed in a zinc-deficient nutrient solution. No deficiency symptoms apparent*

Treatment	Mean dry weight, in gm.	Zinc content, in p.p.m.
Zinc-deficient solution:		
Sprayed with zineb	14.9	19.9
Sprayed with zinc sulfate	18.0	23.2
Unsprayed	6.4	3.1
Complete solution:		
Unsprayed	19.7	27.0
L.S.D., 5 per cent level,	3.1	0.9
1 per cent level,	4.2	1.2

[3]Equivalent to 2 lb. Dithane Z-78 in 100 gal. water, which is the concentration commonly used on tomatoes for disease control in the field.

[4]Equivalent to ½ lb. commercial zinc sulfate in 100 gal. water.

these two treatments at the five percent level (Table 1). Growth expressed on a dry-weight basis was best in unsprayed plants maintained in a complete nutrient solution. The concentration of zinc in the unsprayed terminal growth of all plants appeared to be directly correlated with total dry weight of the plants. However, the concentration was significantly higher in plants sprayed with zinc sulfate than in those receiving zineb.

Plant growth in series 2, as in the preceding series, was greatest in unsprayed plants in a complete nutrient solution (Table 2). Growth increment or recovery following spraying was essentially the same for plants sprayed with zineb and those receiving zinc sulfate. Zinc-deficiency symptoms (darkening of the bases of the petiolules, epinasty, yellow mottling, and necrotic spotting of the leaflets), which were pronounced in this series at the time of spraying, did not appear in the subsequent new growth.

TABLE 2.—*Mean dry weight and growth increment of tomato plants sprayed with zineb and zinc sulfate 25 days after being placed in a zinc-deficient nutrient solution. Severe deficiency symptoms apparent*

Treatment	Mean dry weight, in gm.	Growth increment in 20-day period after spraying	
		Inches	Percentage
Zinc-deficient solution:			
Sprayed with zineb	21.7	12.4	157
Sprayed with zinc sulfate	19.4	13.4	167
Not sprayed	14.9	5.9	72
Complete solution:			
Not sprayed	23.5	9.7	84
L.S.D., 5 per cent level,	3.0	2.5	
1 per cent level,	4.1	3.4	

There was no significant weight difference in series 3 between plants sprayed with zineb and those sprayed with zinc sulfate (Table 3). Both sets were significantly better than nonsprayed plants grown in a complete nutrient solution.

Chemical and biological assay of technical zineb.—In the washing experiments, water-soluble zinc removed at the end of each of the first four series (20 filtrations) amounted to 10 p.p.m. At the end of the fifth series the amount of water-soluble zinc had dropped to 5 p.p.m. and stayed at this level through the remaining five washings (25 filtrations).

In experiments comparing unwashed technical zineb with the washed compound and with the laboratory-prepared fungicide, no significant differences in dry weight of sprayed plants were found at the end of 40 days.

DISCUSSION.—Tomato plants sprayed with a zineb fungicide absorb a measurable amount of zinc from this compound. The amount they absorb from one application in the absence of any other form of zinc is enough to maintain a growth rate comparable to that of plants sprayed with zinc sulfate. The zinc in zineb is apparently somewhat more available to or more readily absorbed by the plant than that in ziram and zinc 8-quinolinolate.

TABLE 3.—*Mean dry weight of tomato plants sprayed with zineb and zinc sulfate 18 days after being placed in a complete nutrient solution. No deficiency symptoms apparent*

Treatment	Mean dry weight, in gm.
Complete solution: Sprayed with zineb	23.03
Sprayed with zinc sulfate	23.5
Unsprayed	20.3
Zinc-deficient solution: Unsprayed	15.5
LSD at 5 per cent level	1.0
at 1 per cent level	1.6

These data show that technical zineb is slightly soluble in water up to approximately 5 p.p.m. Partial confirmation of this is furnished by data from another source.[5] Thus it might be expected that a relatively small but fairly constant supply of zinc would be available to plants sprayed with this fungicide as long as a water film were present on the foliage. In the field the amount of available zinc from this source would be conditioned largely by wash-off by rains and possible decomposition due to weathering.

A recent report[5] indicates that the residue from spraying nabam and zinc sulfate on tomato fruit in the laboratory may be embedded in the cutin and therefore very difficult to remove by scrubbing in water. This may account for the zinc found in samples of fruit sprayed with this fungicide in the present experiments. Therefore, the results of the fruit analyses for zinc may have been more a measure of surface retention than one of storage within the fruit. In any event it is doubtful that the differences in zinc content of the fruit are analytically significant.

The chemical and biological assays of technical zineb show that there is little if any water-soluble zinc present in this compound as a residue from its manufacture. Had any such fractions been present they should have been detected in the first series of washings and filtrations. The comparative bioassay of unwashed technical zineb and laboratory-made zineb prepared by using an excess of nabam is significant in this connection. Any difference in the inorganic zinc content of technical zineb and of zineb formulated for spraying or dusting might be traceable to small amounts of this metal in the diluent or carrier.

It is extremely likely that increasing the concentration of zinc sulfate or zineb in these experiments would

[5] "The determination of Dithane in plants," by H. B. Jones and H. Baum, and presented by Dr. E. L. Stanley, Rohm and Haas Company, as Exhibit 1016 at the Food and Drug Administration Residue Tolerance Hearings, May, 1950, in Washington, D. C. Acknowledgment is made of the courtesy of Mr. Gordon Brandes, Rohm and Haas Company, in supplying the authors with a copy.

have resulted in increased growth over that obtained. There is no evidence that optimum concentrations of these two chemicals ever were used either in the nutrient solutions or as sprays. The only bases for calculation of dosage levels of zinc sulfate for the nutrient cultures were the concentration in Hoagland's solution and, for the sprays, those concentrations reported by investigators who have sprayed field-grown tomatoes.

Zinc would appear to have been the principal element in zineb utilized by the plants in these experiments. Equivalent growth responses from zinc sulfate and zineb indicate that the nitrogen, carbon, and sulfur in zineb were not used to any appreciable extent where zinc was the limiting factor. However, it is conceivable that where zineb and related organic compounds are sprayed several times on plants in the field, enough nitrogen might be applied to have a significant effect on growth. Palmiter (10) has pointed out such a possibility from the use of ferbam (ferric dimethyl dithiocarbamate) on apple trees growing in nitrogen-deficient soils.

SUMMARY

Zinc analyses were run on stems and fruit of tomato plants sprayed in the field with several organic zinc fungicides. The highest concentrations of this element in stems were found in general in plants receiving zineb (zinc ethylene bisdithiocarbamate). The results of fruit analyses were inconclusive.

Comparative zinc concentrations and growth rates were determined for young tomato plants grown in a zinc-deficient and a complete Hoagland's solution and sprayed once with a suspension of technical-grade zineb and a solution of zinc sulfate. In certain experiments the plants were sprayed before zinc-deficiency symptoms appeared, and in others only after such symptoms were evident. Although significantly larger quantities of zinc were found in plants sprayed with zinc sulfate than in those sprayed with zineb, there was no difference between the two in growth rate. Both zineb and zinc sulfate resulted in significant growth rate increases when sprayed on plants grown in a complete Hoagland's solution.

Chemical and biological assays of technical zineb did not reveal the presence of inorganic zinc as a residue from manufacture. Zineb was found to be soluble in water up to 5 p.p.m.

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THE PHOSPHATE EFFECT IN PLANT VIRUS INOCULATIONS[1]

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The effect of K_2HPO_4 and Na_2HPO_4 in increasing infection with tobacco mosaic virus on bean was clearly set forth by Thornberry (16). Stanley (13) has confirmed this and noted that the effect was much less when *Nicotiana glutinosa* was used as the test host. Though Thornberry and Stanley pointed out the possibility that the phosphate effect might be on the virus or on the host, subsequent workers (2,4,5,9, 15) seem to have believed or assumed that the effect was on the virus. Evidence that the effect is on the host rather than on the virus is presented herein. Also, the magnitude of the phosphate effect as observed in this study is much greater than reported by Thornberry and Stanley.

METHODS.—The standard method of inoculation was to dust the host leaves with carborundum as described by Rawlins and Tompkins (12) and then to apply a virus-water inoculum to the carborundum-dusted surface by means of a brush as used by Takahashi (14). The inoculum was prepared by grinding in a mortar a small measured piece of an infected leaf (usually about 1 sq. cm.) with a small amount of water (usually 2 drops) and then diluting as desired. From the known green and dry weight per unit area of the tissues used, the tissue concentration of the virus inoculum can be calculated readily. For example, tobacco leaves as used here averaged about 3 gm. green weight and 0.2 gm. dry weight per sq. decimeter. Therefore, 1 sq. cm. of green leaf tissue ground and diluted to 3000 cc. would give a 0.001 per cent tissue concentration. By the brushing method used about 0.0036 cc. of inoculum was applied per sq. cm. of inoculated leaf surface. From the reported virus concentration (1) of systemically infected tobacco, which was the source of virus for most of the experiments reported herein, from the known distribution of inoculum as given above, and from the infections per unit area as given later, the absolute efficiency of the inoculation methods can be estimated if desired.

The tobacco mosaic virus used was a common strain obtained from W. N. Takahashi and the tobacco ringspot virus was a yellow strain from Glenn S. Pound. Tobacco necrosis and cabbage black ring viruses were from C. M. Tompkins. The alfalfa mosaic virus was a Ladino clover strain from S. P. Swenson. The white clover mosaic virus was taken from naturally infected white clover, and was not identified in terms of Johnson's (8) two components of this virus. Ordinary cucumber mosaic virus and beet mosaic viruses were obtained from E. S. Sylvester. The pea mosaic and broad-bean mosaic viruses were taken direct from naturally infected plants, but from their symptoms on several varieties of beans they appeared distinct from each other and from all other viruses listed above. Potato virus X was taken from leaves of Netted Gem plants.

Twin-leaf or half-leaf methods (6,11) were used in most cases for comparison of virus concentrations or for comparison of inoculation methods. Primary unifoliate leaves of bean (*Phaseolus vulgaris* L.) were used and half-leaves of these twin-leaves afforded four very closely comparable half-leaf units. Bean leaves about 36 sq. cm. in area usually were inoculated 8 to 12 days after the seed was planted, and the inoculated leaves were still growing at the time of inoculation. When the number of lesions was large only those on representative sq.-in. or sq.-cm. areas were counted.

RESULTS.—*Host injury from phosphate.*—Carborundum-dusted bean leaves were rubbed with 1, 3, and 10 per cent K_2HPO_4 as in virus inoculations. Some leaves were washed with water 2 min. after treatment and some were not. The condition of the leaves was noted one day later. Injury resulted on all unwashed leaves and was roughly proportional to the phosphate concentration. Leaves rubbed with 10 per cent K_2HPO_4 were killed. On plants washed within 2 min., injury was apparent only on the leaves rubbed with 10 per cent K_2HPO_4. In inoculations with virus solutions containing phosphate the leaves were usually washed at the end of an experimental series, or about 10 min. after the start of inoculation. In some cases, especially in hot weather, the leaves were injured, presumably from the phosphate, since similar treatments without phosphate usually caused no injury. Undoubtedly the phosphate injury began immediately on application and its extent was determined by the condition of the plant, the environment, and the time of contact with the leaf.

Phosphate effective with several viruses attacking bean.—The effect of 1 per cent K_2HPO_4, hereafter referred to as phosphate, on the number of local lesions produced by several viruses is shown in Table 1, the visual effect of phosphate on infection with tobacco mosaic virus is illustrated in Fig. 1, and the quantitative effect of various dosages of phosphate on three viruses is illustrated diagrammatically in Fig. 2. The striking feature of the data is that infection by the nine viruses that caused local lesions on bean greatly increased when phosphate was added to the inoculum. When these same viruses were inoculated to other local-lesion hosts (tobacco mosaic virus on *N. glutinosa;* tobacco necrosis virus on tobacco, beet, cucumber, and sunflower; and cabbage black ring virus on tobacco), the increase in infection due to phosphate was much less than on bean. With potato virus X and beet mosaic virus, which did not give

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TABLE 1.—*Effect of phosphate on virus inoculations*

Virus and source host	Local-lesion host	Percentage tissue concentration in inoculum	Number of local lesions	
			No phosphate in inoculum	1 per cent K_2HPO_4 in inoculum
Tobacco mosaic—*Nicotiana tabacum*	*Nicotiana glutinosa*	0.001	324	585
Do	*Phaseolus vulgaris, Pinto variety*	0.001	6	1410
Tobacco ringspot—*Nicotiana tabacum*	*N. tabacum*	0.1	3	80
Do	Pinto bean	0.1	0[a]	44
Tobacco necrosis—*Nicotiana tabacum*	*N. tabacum*	0.05	16	52
Do	10 bean varieties	0.05	51	595
Do	*Cucumis sativus*	0.05	11	15
Do	*Beta vulgaris* (sugar beet)	0.05	38	41
Do	*Helianthus annuus*	0.05	65	93
Potato virus X—*Solanum tuberosum*	*Gomphrena globosa*	0.02	38	40
Alfalfa mosaic—*N. tabacum*	Pinto bean	0.01	3	44
White clover mosaic—Bountiful bean	10 bean varieties	0.02	31	198
Do	*Vigna sinensis*	0.05	8	259
Cucumber mosaic—*N. glutinosa*	Rusted Pinto bean	0.05	0	9
Beet mosaic—Garden beet	Garden beet	0.1	79	60
Pea mosaic—*Pisum sativum*	Pinto bean	0.1	2	11
Broad bean mosaic—*Vicia faba*	Bountiful bean	0.1	9	65
Cabbage black ring—*Brassica oleracea* var. *botrytis*	Pinto bean	0.1	21	64
Do	*N. tabacum*	0.1	58	96

[a] Infection of Pinto bean without phosphate has occurred in other trials.

local lesions on bean with or without phosphate, the use of phosphate resulted in no apparent increase in infection on appropriate local-lesion hosts. This specificity of phosphate effect strongly indicates that the phosphate acts on the host rather than on the virus.

Use of detached leaves.—The effect of phosphate in increasing virus infection was studied mostly on attached leaves of greenhouse plants, and all data of Table 1 and Figures 2 and 3 are for such leaves. The phosphate effect also was demonstrated on detached leaves on water or on 8 per cent sucrose solution (Fig. 1). Six days after inoculation, tobacco mosaic virus lesions on greenhouse plants averaged 0.32 mm. in diameter; lesions on detached leaves on water at 31° C. averaged 1.35 mm., and lesions on detached leaves on 8 per cent sucrose averaged 0.12 mm. Although lesions were large on detached leaves on water, these infected leaves rapidly decayed. The lesions on

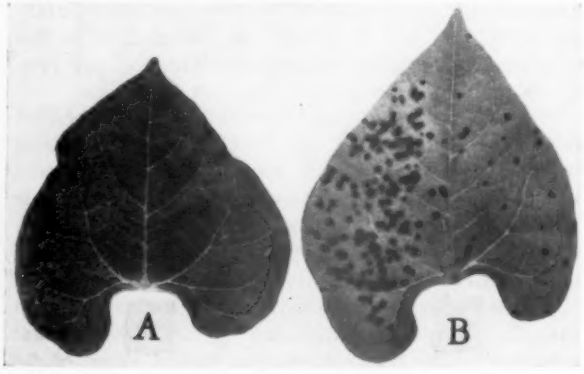

FIG. 1. Effect of phosphate on inoculation with tobacco mosaic virus on bean. The right side of each leaf was inoculated with 0.005 per cent tissue suspension of mosaic tobacco leaf tissue in water. The left side was inoculated with 0.005 per cent tissue in 1 per cent aqueous K_2HPO_4. The leaves were detached after being rinsed and dried. Leaves were incubated at 31° C., leaf A on 8 per cent sucrose, leaf B on water. Photographed March 15, 1951, 4 days after inoculation. (Photograph by Victor Duran.)

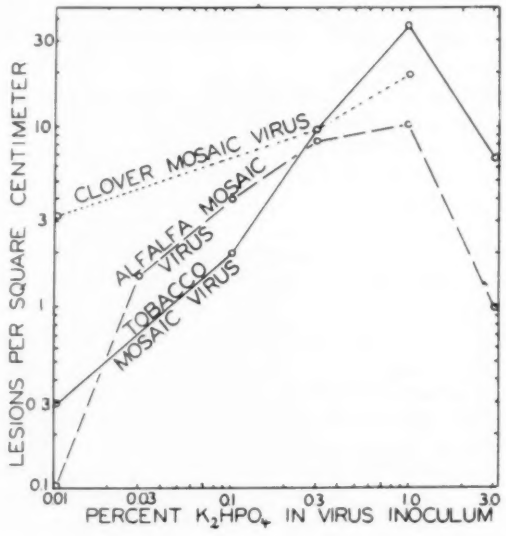

FIG. 2. Effect of concentration of phosphate in the inoculum on the number of virus lesions formed on inoculated bean leaves by three viruses. The concentration of diseased host tissue used as inoculum was as follows: 0.001 per cent for tobacco mosaic virus (March 16, 1951), 0.05 per cent for white clover mosaic virus (March 5, 1951), and 0.01 per cent for alfalfa mosaic virus (March 3, 1951). The pH of the suspensions was 9 throughout. The optimum concentration of phosphate was about 1 per cent. (Photograph by L. V. Messier.)

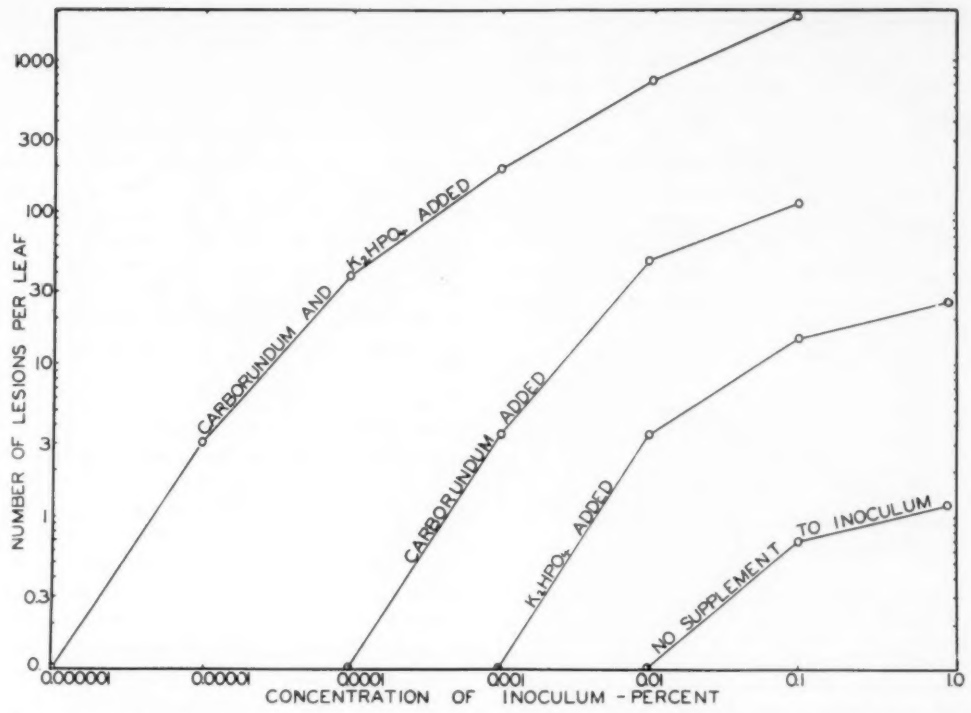

FIG. 3. Relation of phosphate and carborundum to infection of Pinto bean with tobacco mosaic virus inoculum containing 0.1 per cent tissue. The combined effect of carborundum and phosphate is to increase about 2800-fold the number of lesions or to decrease to 1/100,000 the amount of inoculum necessary to produce one lesion per leaf, as compared to inoculations without phosphate or carborundum. Average results of 11 trials, March 25 to June 7, 1951. (Photograph by L. V. Messier.)

leaves on sucrose solution are difficult to count or measure without magnification.

Varietal susceptibility to phosphate effect.—Preliminary results indicate that the increase in infection due to phosphate is greatest on bean varieties which are resistant to infection without phosphate. Results with tobacco mosaic virus and tobacco necrosis virus (Table 2) and the bean varieties Michelite and White Blue Lake illustrate the effect well. Phosphate increased infection fourfold in Michelite, a variety susceptible to tobacco mosaic in the absence of phosphate. It increased infection 218-fold in White Blue Lake, a variety resistant to the virus in the absence of phosphate. More evidence is needed to show whether this generalization is a safe one.

TABLE 2.—*Phosphate effect in virus inoculations on bean varieties*[a]

Virus inoculum and bean variety	Lesions per leaf	
	Without phosphate	With phosphate
Tobacco mosaic		
Navy	4.0	25.0
Michelite	11.5	57.0
White Blue Lake	0.5	109.5
Pink	4.0	69.0
Creaseback	4.5	55.5
Pinto	2.0	61.0
Golden Cluster	0.0	266.0
Brown Kentucky Wonder	0.0	42.0
Tobacco necrosis		
Pinto	6.0	53.0
Black Blue Lake	3.0	118.0
Bountiful	12.0	68.0

[a] All inoculations with carborundum; data from trials of March 4 and 20 and June 4, 1951.

Combined action of carborundum and phosphate.—Since carborundum and phosphate are so effective independently in increasing virus infection, trials to measure their separate and combined action under similar conditions were performed (Fig. 3). Carborundum alone was more effective than phosphate but the two together were more effective than either alone or than the effect of the two acting separately. The ratio of the numbers of lesions formed from 0.1 per cent inoculum without supplement to the number formed with 1 per cent K_2HPO_4 added to the inoculum, with carborundum added to the leaf before inoculation, and with both K_2HPO_4 and carborundum was 1:20:165:2860 respectively. The ratio of the amounts of inoculum to produce one lesion per leaf under these conditions was about 100,000:1,000:-100:1 respectively.

Action of phosphate on infection of N. glutinosa.—The effect of age and position on the susceptibility of leaves of *N. glutinosa* to tobacco mosaic was studied in four tests. Results of a typical test involving two

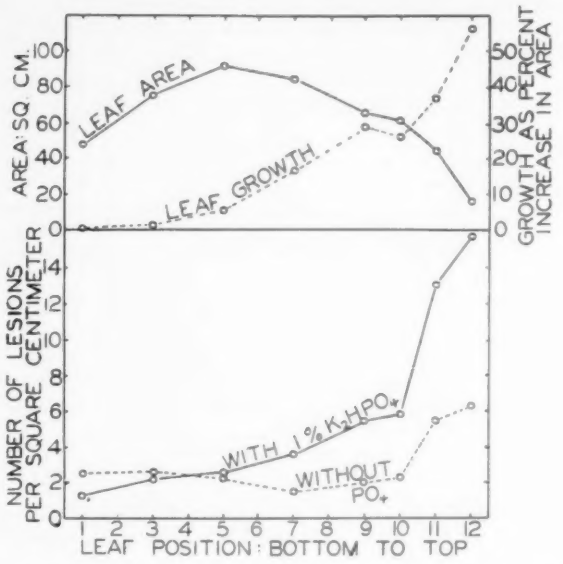

FIG. 4. Effect of phosphate in the inoculum and of leaf position on infection of leaves of *Nicotiana glutinosa* with tobacco mosaic virus. The addition of phosphate to the inoculum decreased infection on the old lower leaves but increased infection on the young upper leaves. Leaves measured and inoculated 12 m., March 14, 1951; leaves remeasured and lesions counted March 17. (Photograph by L. V. Messier.)

plants and 3453 lesions are in figure 4. With 12 leaves of usable and stated size and with 0.001 per cent tissue suspension from systemically infected tobacco, the number of lesions in the control inoculations without phosphate ranged from 1.6/sq. cm. on the central leaves to 2.4/sq. cm. on the lower leaves and 6.2/sq. cm. on the upper leaves. With phosphate, the number of lesions ranged progressively from 1.2/sq. cm. on the lower leaves to 15.7 on the upper leaves. The ratio of number of lesions with phosphate to the number without phosphate ranged from 0.5 on the lower leaves to 2.5 on the upper leaves, with no apparent difference in the ratio from the 7th to the 12th leaf. In another test there were nine times as many lesions on the upper leaf with phosphate as without. The smaller number of lesions with phosphate than without for inoculations on the lower leaves, and the larger number of lesions with than without phosphate for the upper leaves prevailed in all tests. As starting and ending positions of leaves in a test of this type are difficult to define, the actual size of the leaves at the time of inoculation and the measured rate of growth during 3 days are given in figure 4. It would appear that phosphate is most effective in increasing virus infectivity on rapidly growing leaves of *N. glutinosa*.

Pretreatment of test host with phosphate.—If the effect of phosphate were on the host it might be expected that pretreatment of the test host with phosphate would increase its susceptibility to virus. Bawden (1) has shown that enriched phosphate nutrition increases the susceptibility of host plants to virus to a greater extent than did any other single nutrient tested.

In this study only one type of short pretreatment of the host with phosphate has clearly increased the host's susceptibility to virus. Bean leaves were dipped in phosphate solution at 45° C., washed in water, inoculated while wet with 0.001 per cent tissue of mosaic-infected tobacco, and again washed. Controls similarly treated with water at 45° C. were maintained. In two trials, with 2 leaves per treatment in each trial, the average number of local lesions per leaf was as follows: untreated leaves, 2.8; leaves dipped in water at 45° C. for 0.5 min., 5.7, and for 1.0 min., 20.0; leaves dipped in 1 per cent K_2HPO_4 at 45° C. for 0.5 min., 31.0, and for 1.0 min., 63.0. Treatment of leaves with hot water greatly increased the susceptibility of leaves to tobacco mosaic virus, but treatment with hot phosphate caused an even greater increase.

The effect of pretreatment of leaves with hot water or hot phosphate was much less when inoculation was made 6 hr. after treatment than when made within 5 min. of treatment. When phosphate was added to the inoculum, no effect of pretreatment of leaves with hot water or hot phosphate was detected.

Other pretreatments of beans with phosphate before inoculation did not result in clearly increased susceptibility to tobacco mosaic virus: 1) Phosphate was sprayed on the leaves and the surface phosphate was washed off later; 2) leaves were brushed with phosphate plus carborundum and the phosphate was then washed off; 3) stems of detached plants were placed in dilute phosphate and allowed to take up the solution in the transpiration stream; and 4) phosphate was forced into the intercellular spaces by means of a heavy spray of phosphate solution on the lower leaf surface before the upper leaf surface was inoculated.

Time from inoculation to washing.—By analogy from inoculation with fungus and bacterial pathogens of plants, it might be expected that washing the leaf surface immediately after the application of inoculum to that surface would decrease the amount of infection with viruses. Holmes (7), however, has shown that with tobacco mosaic virus on *N. glutinosa*, most of the effective penetrations by the virus occurred at the time the inoculum was rubbed on the leaf surface. Washing the leaves with water immediately after they were inoculated actually increased the number of lesions. This has been confirmed by the writer on bean when no phosphate was added to the inoculum. With 1 per cent K_2HPO_4 in the inoculum, however, washing the leaves immediately after inoculation resulted in much less infection. In one trial the number of lesions on five leaves from which the inoculum and phosphate was washed off as quickly as possible was 84, whereas on the five opposite leaves where the inoculum plus phosphate was left on for 5 min. before washing, the number of lesions was 415.

Time from mixing virus and phosphate to inoculation.—If the action of phosphate in virus inoculations were principally on the host, no effect of length of exposure of virus and phosphate to each other before inoculation would be expected, whereas if the action were on the virus it might be expected that infection would be increased if virus and phosphate were allowed to react for an optimum time before being used as inoculum. In trials to determine this, the number of lesions formed was as great when the virus and phosphate were used immediately after they were mixed as when used at various intervals up to an hour after mixing. This evidence also supports the theory that the action of phosphate is on the host rather than on the virus.

Bean vs. N. glutinosa for local-lesion production.—Despite the advantage of bean over *N. glutinosa* as a local-lesion host for tobacco mosaic virus, as pointed out by Price (11), *N. glutinosa* is perhaps used and preferred by most investigators. This may be because of the greater variability of results on bean when phosphate is not used in the inoculum. The writer believes that when phosphate is used in the inoculum, bean is much superior to *N. glutinosa* as a test local-lesion host for measuring relative concentration of tobacco mosaic virus. In two typical trials with 0.001 per cent suspension of tobacco mosaic tissue as inoculum, the number of lesions per sq. cm. was 0.70 and 0.39 with and without phosphate, respectively, on *N. glutinosa*, and 6.4 and 0.086 with and without phosphate on Pinto bean. Therefore, without phosphate *N. glutinosa* gave five times as many lesions per unit leaf area as bean, but with phosphate bean gave nine times as many lesions as *N. glutinosa*. To the writer, the only remaining advantage of *N. glutinosa* over bean is the greater size of the lesions on *N. glutinosa*.

The following bean varieties have proved to be local-lesion hosts of tobacco mosaic virus: Pinto, Creaseback, Idaho Refugee, Navy, Michelite, White Blue Lake, Pink, Large White, California Small White, Scotia, Brown Kentucky Wonder, and Red Mexican. The following varieties did not produce local lesions: Bountiful, Black Blue Lake, French Horticultural, Red Kidney, Golden Gate Wax, Kentucky Wonder Wax, and White Kentucky Wonder. The variety Pinto has been most extensively used as a local-lesion host in these studies because it was first tested, and because it is also susceptible to bean rust, bean powdery mildew, bean anthracnose, tobacco ringspot virus, tobacco necrosis virus, alfalfa mosaic virus, pea mosaic virus, and white clover mosaic virus.

Virus entry through wounds made before inoculation.—When carborundum-dusted leaves of *N. glutinosa* were rubbed with a brush dipped in water and the leaves then dipped in or sprayed with a virus or virus-plus-phosphate suspension, infection occurred as noted by Kalmus and Kassanis (10). When beans were similarly inoculated very little infection occurred, but if carborundum-dusted beans were rubbed with a dry brush and then dipped in a virus-plus-phosphate suspension, more infection resulted. In one trial leaves of *N. glutinosa* gave 2.0 lesions per sq. cm. when rubbed dry and then dipped in 0.001 per cent tobacco mosaic tissue suspension in 1 per cent phosphate, while leaves brushed wet and then dipped in virus plus phosphate gave 1.5 lesions per sq. cm. In the same trial, carborundum-dusted bean leaves which were rubbed with a dry brush and then dipped in virus plus phosphate gave 3.7 lesions per sq. cm., whereas leaves rubbed wet and then dipped in virus phosphate gave only 0.48 lesions per sq. cm. Control bean leaves dusted with carborundum and then brushed with the same virus plus phosphate gave 18.5 lesions per sq. cm.

It therefore appears that virus can readily enter water-saturated wounds on *N. glutinosa* but not on bean, whereas on bean the virus can readily enter unsaturated wounds. However, inoculation of leaves with unsaturated wounds gave many fewer lesions than when wounding and inoculation were simultaneous as in the standard method.

Discussion.—Several differences between the writer's methods and those of Thornberry (16) and Stanley (13) may aid in explaining the differences in results. The writer's inoculations were made with a brush; those of Thornberry and Stanley were made with a pad. Thornberry used the bean variety Scotia and Stanley used the variety Early Golden Cluster, whereas the writer has used principally the variety Pinto, and it is indicated from trials of several varieties that the greatest increase in infection from use of phosphate results on varieties which give few lesions without phosphate. The writer used carborundum in most trials whereas Thornberry and Stanley did not, and it is shown that the effect of phosphate is much greater when carborundum is used. Lastly, it is shown in this paper that the time elapsing between inoculation and washing of leaves is important. This probably averaged about 10 min. in this study, but details are not given by Thornberry and Stanley.

Evidence that the effect of phosphate may be on the virus has been presented by Thornberry (16), Stanley (13), Takahashi (115), and Black (2). Some of these workers seem to regard the effects of K_2HPO_4 and Na_2HPO_4 as due to their pH which is about 8.5 (16). That the phosphate effect is not entirely a buffer or pH effect is indicated by the data of Stanley (13) which showed that other buffers at pH 7 and 8 were much less effective in increasing infection than were phosphate buffers, and by the data of Thornberry (16) and figure 2 of this paper which show that various concentrations of phosphate with the same pH had greatly different effects. The ratio of phosphate to virus can be estimated from the data in figure 2 and under Methods. In a 0.001 per cent virus tissue suspension plus 1 per cent phosphate, which gave about 200 times as many lesions as the same virus suspension without phosphate, the weight

ratio of phosphate to virus would be about 500,000 to 1, and the molecular ratio (assuming tobacco mosaic virus is a pure protein with a molecular weight of 40,000,000) would be about 100,000,000,000 to 1. At a weight ratio of 50,000 to 1 (0.001 per cent tissue plus 0.1 per cent phosphate), phosphate was practically ineffective in increasing infection. From this finding that a 500,000:1 ratio of phosphate to virus increased infection greatly but a 50,000:1 ratio only slightly, it would seem very unlikely that the effect of phosphate on infection can be due to any chemical interaction between phosphate and virus. The finding of Born et al. (3) that tobacco mosaic virus absorbed only an insignificant amount of radioactivity when allowed to stand in Na_2HPO_4 solution containing radioactive phosphorus also would indicate there is no reaction between virus and phosphate.

That phosphate can increase virus multiplication through its effect as a host nutrient has been pointed out by Bawden (1). However, the nutrient effect seems inadequate as an explanation of the results of this study since Thornberry (16) has shown that $(NH_4)_2HPO_4$ (pH 7.2), NaH_2PO_4 (pH 4.5), and KH_2PO_4 (pH 4.5), which are also plant nutrients, are much less effective than K_2HPO_4 or Na_2HPO_4 as aids in virus inoculations. It seems likely that about 1 per cent phosphate and a pH of about 8.5 are both essential for what is here described as the phosphate effect.

That phosphate increases virus infection because of an effect on the host rather than on the virus is indicated by several independent lines of evidence available from the literature and from this study. There is the parallelism between host injury due to phosphate, and increase in virus infection due to phosphate. As the period of exposure of virus plus phosphate to the host was increased, host injury and virus infection were both increased, though the heaviest infection resulted if the surface virus plus phosphate were removed before apparent host injury resulted.

There also is an apparent similarity between the phosphate effect and the carborundum effect in virus inoculations, and it is generally accepted that the effect of carborundum is on the host, not on the virus. Kalmus and Kassanis (10) have reported that the increase in infection due to abrasives is 5 to 10 times greater on the upper than on the lower leaves of *N. glutinosa*. In the present study it was found that the phosphate effect also is much greater on the upper than on the lower leaves of *N. glutinosa* (Fig. 4).

Another similarity in the phosphate effect and the carborundum effect was the relatively slight increase in infection if the host was treated with carborundum or phosphate before the virus was applied, as compared to simultaneous application of carborundum plus virus or phosphate plus virus. In tests on *N. rustica* without carborundum or phosphate, Holmes (7) also concluded that the virus does not readily enter wounds if these are made before the application of the virus.

The phosphate effect was apparent only on certain hosts, especially beans, but resulted with all viruses tested which gave local lesions on these hosts. Bean varieties differed greatly in their response to phosphate. Therefore, the phosphate effect was host-specific but not virus-specific.

The hypothesis advanced here to explain the phosphate effect is that phosphate increases by chemical action the size, number, and permeability of the perforations in the cells made or started by rubbing the leaves with or without carborundum. These perforations must be large enough to let the virus enter, but so small that the penetrated host cell will not be killed. This is basically the same explanation as that of the carborundum effect advanced by Rawlins and Tompkins (12).

SUMMARY

Solutions of K_2HPO_4 were injurious to bean leaves and the injury increased with increasing time of exposure and increasing concentration of phosphate. The addition of 1 per cent K_2HPO_4 to the inoculum of tobacco mosaic virus, tobacco ringspot virus, tobacco necrosis virus, alfalfa mosaic virus, white clover mosaic virus, broad bean mosaic virus, cucumber mosaic virus, pea mosaic virus, and cabbage black ring virus increased the number of lesions formed on inoculated bean leaves. On other hosts of the above or other viruses the effect of phosphate was much less than when bean was used as the host, and on cucumber, sugar beet, sunflower, and *Gomphrena globosa* no clear increase in virus infection from the use of phosphate was detected. The optimum phosphate concentration for inoculations of tobacco mosaic virus, tobacco necrosis virus, alfalfa mosaic virus, and white clover mosaic virus on bean leaves was about 1 per cent.

On Pinto bean inoculated with 0.1 per cent tobacco mosaic tissue in suspension, the ratio of the number of lesions formed without supplement to the number formed with 1 per cent K_2HPO_4 added to the inoculum, with carborundum added to the leaf before inoculation, and with both carborundum and K_2HPO_4 was 1:20:165:2860 respectively. The ratio of the amounts of inoculum to produce one lesion per leaf under these same conditions was about 100,000:1,000:100:1.

The addition of phosphate to tobacco mosaic virus inoculum decreased infection on the lower leaves of *Nicotiana glutinosa* but increased infection on the upper leaves. Without phosphate in the inoculum more lesions of tobacco mosaic virus were formed per unit area of leaves on *N. glutinosa* than on bean, but with phosphate in the inoculum nine times as many lesions were formed on bean as on *N. glutinosa*.

The effect of phosphate in increasing infection of tobacco mosaic virus on bean was demonstrated on potted plants, on detached leaves on water, and on detached leaves on sucrose solution. On detached leaves on water the lesions were much larger than on

greenhouse plants, but on detached leaves on 8 per cent sucrose the lesions were much smaller than on greenhouse plants.

Dipping bean leaves for 1 min. in 1 per cent K_2HPO_4 at 45° C. greatly increased their susceptibility to tobacco mosaic virus. Treatment with hot water also increased susceptibility of bean leaves, but to a much lesser extent than did treatment with phosphate.

When bean leaves inoculated with a water suspension of tobacco mosaic virus were washed with water immediately after inoculation, no decrease in infection resulted in comparison with unwashed leaves, but when bean leaves inoculated with a phosphate-plus-virus suspension were washed immediately after inoculation the amount of infection was much less than when the washing was delayed up to 30 min.

On bean leaves very little infection resulted if the carborundum-dusted leaves were rubbed with a wet brush and then dipped in a suspension of tobacco mosaic virus plus phosphate, but abundant infection resulted if the carborundum-dusted leaves were rubbed with a dry brush before being dipped in a suspension of virus plus phosphate.

From the original data presented and from data in the literature it is believed that the increased infection of viruses following addition of phosphate to the inoculum is due largely to the effect of phosphate in increasing the susceptibility of the host rather than in increasing the infectivity of the virus. The hypothesis is advanced that phosphate increases susceptibility by its chemical action in increasing the size and number of cell perforations started by the act of inoculation by rubbing the leaf surface.

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TWO NEW PHYTOPHTHORA DISEASES OF ORNAMENTAL HIBISCUS [1]

M. O. James and A. G. Plakidas

Two species of ornamental hibiscus (*Hibiscus rosa-sinensis* L. and *schizopetalus* L.) have become popular flowering shrubs in Louisiana and other Southern States (3). This paper describes two apparently new diseases of hibiscus, a foot rot and a leaf and stem blight, which in recent years have caused considerable concern and losses to nurserymen and home gardeners. Both diseases are caused by species of *Phytophthora*, and both have been observed in several nurseries and localities in Louisiana. The *Phytophthora* causing foot rot (4) fits well the description of *P. cactorum* var. *applanata* as given by Chester (2), while the cause of leaf and stem blight was identified as *P. palmivora* Butler. Foot rot was found in nature on practically every variety of *H. rosa-sinensis* and *H. schizopetalus* grown in Louisiana; leaf and stem blight was observed on fewer of the most popular varieties.

No report of *Phytophthora* on *H. rosa-sinensis* or *H. schizopetalus* could be found in the literature, but Phytophthoras have been reported on other species of hibiscus: *P. parasitica* on *H. manihot* in Japan (9) and on *H. sabdariffa* and *H. cannabinus* in Java (7), and *Phytophthora* sp. on *H. sabdariffa altissima* in Malaya and Africa (5, 10). A species of *Nectria* was associated with a serious foot rot of *H. schizopetalus* in the Philippines (1). Small reported *Rhizoctonia bataticola* on the foliage of *H. rosa-sinensis* in Ceylon (8). Dickey (3) in Florida refers to varieties tolerant to the "root-rot" fungus but does not identify the organism.

DESCRIPTION OF THE DISEASES.—*Foot rot.*—The first symptom to appear in the field is a yellowing of the lower leaves, which may persist for 2 or 3 days before the plant completely wilts. On removing the infected plants from the soil, one can observe the characteristic water-soaked, discolored area of the foot of the plant (Fig. 1). The discoloration occasionally occurs just above or below the soil line, as in figure 2, A, where infection appears to have occurred through the uppermost root. Isolations from the margins of discolored areas have yielded a *Phytophthora*.

Leaf and stem blight.—Early in the course of the foot-rot investigations, a leaf and stem blight disease appeared on hibiscus plants that were heavily syringed in the propagation bed. While plants were being vegetatively propagated by marcottage (air layering), dark, water-soaked areas appeared on the leaves and stems above and around the sphagnum wraps. These water-soaked areas spread rapidly over the leaves and stems, and the plants were killed in a very short time. When blighted leaves were examined microscopically, dense tufts of papillate sporangia were on the under surfaces. Isolations from blighted stems and leaves yielded a *Phytophthora* quite different from the foot-rot organism.

FIG. 1. Foot rot of ornamental hibiscus. Lower portion of naturally infected plant showing characteristic water-soaked, discolored area at base of plant.

CAUSES OF THE DISEASES.—Isolations from foot-rot material consistently yielded a *Phytophthora* which was identified as *P. cactorum* var. *applanata* (2, 6). The fungus was obtained easily in culture by washing diseased material in running water for 15–24 hr., carefully removing tissue from the periphery of the discolored areas, and plating directly to oatmeal agar.

P. cactorum applanata produced abundant nonpapillate sporangia and occasional oospores on oatmeal agar. Oogonia and paragynous antheridia were obtained by transferring mycelium from pea broth to distilled water. The sporangia averaged 20.04 ± 2.85 × 20.41 ± 2.13 μ, the extremes being 18.50 × 31.50 μ. It grew well on oatmeal, malt-extract, and potato-dextrose agars at 7° C., but did not grow at 35° C. It was pathogenic to tomato, apple, and pear fruits and nonpathogenic to potato tubers.

Isolations from the leaf and stem blight material yielded a *Phytophthora* which was identified as *P. palmivora* Butler (6, 11). Isolations from the blight material were made in the same manner as were the foot-rot isolations.

[1] Accepted for publication October 28, 1951.

P. palmivora produced in culture only papillate sporangia which averaged 43.25 ± 7.14 × 30.56 ± 3.26 μ. It grew well at 35° C. on oatmeal, malt-extract, and potato-dextrose agars, but no growth occurred at 7° C. and only sparse growth at 15° C. It was pathogenic to tomato, apple, and pear fruits, and also very pathogenic to potato tubers, producing a putrid brown rot.

Occasionally, *Colletotrichum* sp. was encountered fruiting on dead stems of plants affected with the leaf and stem blight. The fungus was obtained in pure culture and its pathogenicity tested on healthy plants. It did not cause infection, and it is therefore considered a secondary invader of tissues killed by the *Phytophthora*.

INOCULATIONS.—The varieties of hibiscus used in the inoculation experiments were: Kona, Minerva, Peachblow, Brillantissima, Agnes Gault, Jigora, Euterpe, Columbia, Fair Jeanette, Golden Dawn, Indian Chief, White Hawaiian, Psyche, and Schizopetalus.

The methods used in inoculation of hibiscus plants with *P. cactorum applanata* and *P. palmivora* were as follows:

1. Wound inoculation at crown. Bits of mycelium were placed in wounds at the crown of the plant, and the wounds were wrapped with moistened cotton.

2. Infestation of soil. Both sterilized and non-sterilized soil in 6-in. pots was mixed with oat cultures of the respective fungi, a pint of inoculum per pot.

3. Leaf inoculation. Disks of mycelium from Petri plates were placed directly on the surface of hibiscus leaves.

4. Zoospore infestation. This method was used only with *P. palmivora*, in which zoospore production was obtained by adding 20 cc. of sterile water to a Petri plate of the actively growing fungus. After 20 min., the water from the Petri plates was collected and put into a funnel which was connected by a series of rubber and glass tubing so that the liquid flowed evenly at the base of the plant into the potted soil. Soil was infested in this manner at an estimated rate of 2000 zoospores per plant.

A limited number of plants was inoculated also by spraying the foliage with zoospores.

The results of inoculation are summarized in table 1. Figure 2, B and C, illustrates the disease produced by inoculations with *P. palmivora*.

Infesting the soil, whether autoclaved or not, with cultures on oats proved the most successful method of inoculation, for symptoms of foot rot and leaf blight appeared on hibiscus plants within 5 to 6 days. Superficially, the symptoms on the plants inoculated with either species of *Phytophthora* were similar; the plants first wilted, then died. On closer examination, it was evident that the foot of the plant usually was infected first by *P. cactorum* var. *applanata*, while *P. palmivora* first infected the leaves and stems and the above-ground parts, but seldom attacked the roots.

In general, inoculation at the wounded crown of the plant was not a very satisfactory method of inoculation; 31 per cent infection with *P. cactorum applanata* and 14 per cent infection with *P. palmivora* was obtained.

TABLE 1.—*Results obtained from several methods of inoculation of hibiscus plants with Phytophthora cactorum applanata and P. palmivora*

Method	Number of plants inoculated—number infected, with	
	P. cactorum applanata	*P. palmivora*
Wound inoculation at crown	16— 5	36— 5
Infestation of autoclaved soil with oat culture	94— 94	71— 58
Infestation of nonautoclaved soil with oat culture	60— 60	36— 36
Leaf inoculation with agar disks	36— 0	36— 36
Zoospore infestation of autoclaved soil	— —	26— 11
Zoospore infestation of nonautoclaved soil	— —	14— 0
Totals	206—159	219—146

FIG. 2. A. Foot-rot infection of ornamental hibiscus, Florida Gem variety, occurring through the uppermost root. B. Two plants of Euterpe variety; the one at right has been killed by wound inoculation at the base of the stem with *P. palmivora*. C. Results of zoospore infestation of sterilized soil. Left to right, variety Indian Chief, check, and inoculated; variety White Hawaiian, check, and inoculated.

Zoospore infestation of autoclaved soil gave 42 per cent infection. The results obtained from zoospore inoculations can be seen in figure 2, C.

When plants were inoculated by placing disks of agar culture of *P. cactorum applanata* and *P. palmivora* directly on the leaves, only *P. palmivora* proved pathogenic. Dark, water-soaked areas appeared on the inoculated leaves 3–4 days after inoculation.

SUMMARY

Two new and apparently undescribed diseases of the ornamental hibiscus, *H. rosa-sinensis* L. and *H. schizopetalus* L., are described. One of these is a foot rot, the other a leaf and stem blight.

The cause of foot rot was a *Phytophthora* which morphologically and culturally fits the description of *P. cactorum* var. *applanata* Chester. The cause of the leaf and stem blight was another species of *Phytophthora* which has been identified as *P. palmivora* Butler.

Both diseases were reproduced by artificial inoculation with pure cultures of the respective fungi on both autoclaved and nonautoclaved soil. Fourteen varieties of hibiscus were used in the inoculation experiments, and all were susceptible to both pathogens.

Foot rot was found to be widespread in Louisiana both in nurseries and in private gardens. Leaf and stem blight, on the other hand, was found chiefly in greenhouses and lath houses in nurseries where vegetative propagation by marcottage (air layering) was practiced and where high humidities were maintained by frequent syringing.

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PATHOLOGY OF CAMELLIA LEAVES INFECTED BY EXOBASIDIUM CAMELLIAE VAR. GRACILIS SHIRAI [1]

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During several recent years a disease caused by *Exobasidium camelliae* var. *gracilis* Shirai, involving the young leaves of the current season's growth, has been noted on *Camellia sasanqua* Thunb. on the campus of Duke University, Durham, North Carolina. Symptoms, evident during March, April, and May, are first noted when the leaf buds begin to open. Infected leaves are greatly enlarged, attaining an area three to five times as great as normal leaves and a thickness of approximately 2 mm. Coincidentally with sporulation by the pathogen, the entire lower surface of the leaf sloughs off as a membrane, and finally the diseased leaves are shed.

Comparison of these findings with those of previous studies of the pathology of other species of *Exobasidium* indicates that none is entirely like that upon Camellia. The previous investigations include those of Petri (5) on *E. discoideum* Ell. and of Eftimiu and Kharbush (1) on *E. discoideum*, *E. vaccinii myrtilli* (Fkl.) Wor., *E. vaccinii uliginosi* Boud., *E. rhododendri* Cram., and *E. uvae-ursi* (Maire) Juel. According to these workers, the mycelium of each of these pathogens is composed of very tenuous intercellular hyphae, approximately 1–1.5 μ in diameter. Certain hyphae aggregate immediately beneath the epidermis prior to formation of basidia, and the basidia terminate hyphae which penetrate the leaf surface between the epidermal cells. Other features common to these diseases include pronounced hypertrophy, lack of complete differentiation of the mesophyll, and reddish discoloration arising from the accumulation of anthocyanin pigment.

The present account of the pathology of *E. camelliae* var. *gracilis* is regarded as an addition to knowledge of diseases induced by Exobasidium and may be presumed to have bearing upon other diseases of the hypertrophic type.

MATERIALS AND METHODS.—The material upon which this report is based was collected in the spring of 1951. Both healthy leaves and infected ones in several stages of the disease were fixed in formalin-acetic-acid-alcohol, dehydrated by passage through a graded series of alcohols, cleared with xylol, and embedded in paraffin. Sections were cut at a thickness of 10 μ and were stained with Haidenhain's iron-alum hematoxylin.

To obtain the pathogen in pure culture, affected leaves were surface-sterilized with 1:1000 mercuric chloride, and leaf fragments were planted upon plates of malt agar. Isolations were also made by removing the membrane from the lower leaf surface and with a needle transferring mature basidiospores to malt agar plates. Within a week grayish to brownish, rough, yeastlike colonies had developed.

OBSERVATIONS.—Identification of fungi in the genus Exobasidium rests mainly upon host specificity. In 1896 Shirai (6) noted on the flowers of *Camellia japonica* L. a fungus which he described as *Exobasidium camelliae*. The one on the leaves of *C. sasanqua* was designated as var. *gracilis*. Apparently he did not make reciprocal inoculations, and none were attempted in North Carolina. Flowers of *C. sasanqua* have never been observed by us to be attacked, nor has an Exobasidium disease been noted on the flowers or foliage of *C. japonica* growing near by.

E. camelliae var. *gracilis* remains dormant within the developing buds from one spring until the next. The renewal of bud growth coincides with renewal of activity by the pathogen, and typically all of the leaves on a new shoot are involved. Occasional leaves were noted in which part of the leaf was swollen and the remainder was apparently unchanged. Within 3 to 4 weeks after the initiation of growth the affected foliage becomes dry and is shed.

Noninfected leaves of *C. sasanqua* (Fig. 1, A) are about 250 μ thick. Both the lower and upper epidermis are thickly cutinized. There is a double layer of palisade cells above a spongy mesophyll, the cells of which tend to be isodiametric and are separated by large air spaces. An infected leaf (Fig. 1, B) is strikingly larger and thicker. Not only are the individual cells much larger, but they are more numerous, indicating that both hypertrophy and hyperplasia have occurred. The two layers of palisade cells, which were so prominent in the normal leaf, may no longer be distinguished. With the exception of the epidermis, the tissues of the leaf present a homogeneous and undifferentiated appearance.

The mycelium, consisting of very delicate hyphae, grows intercellularly within the mesophyll. In preparation for the development of the hymenium it becomes aggregated in the large air spaces, removed by some four or six layers of cells from the lower epidermis (Fig. 1, B). As the basidia elongate (Fig. 1, C), the pressures result in the sloughing off of the superposed layer, consisting of the lower epidermis and four to six contiguous layers of mesophyll cells (Fig. 1, D), so that the basidia eventually complete their development on the exposed surface and discharge their spores to the air. The basidia (Fig. 1, E) are slender, taper uniformly, and bear four curved basidiospores, 11–14 × 2.5–5 μ, that are apically broadened.

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We gratefully acknowledge the help of Wirt H. Wills in preparing the slides for the histological studies.

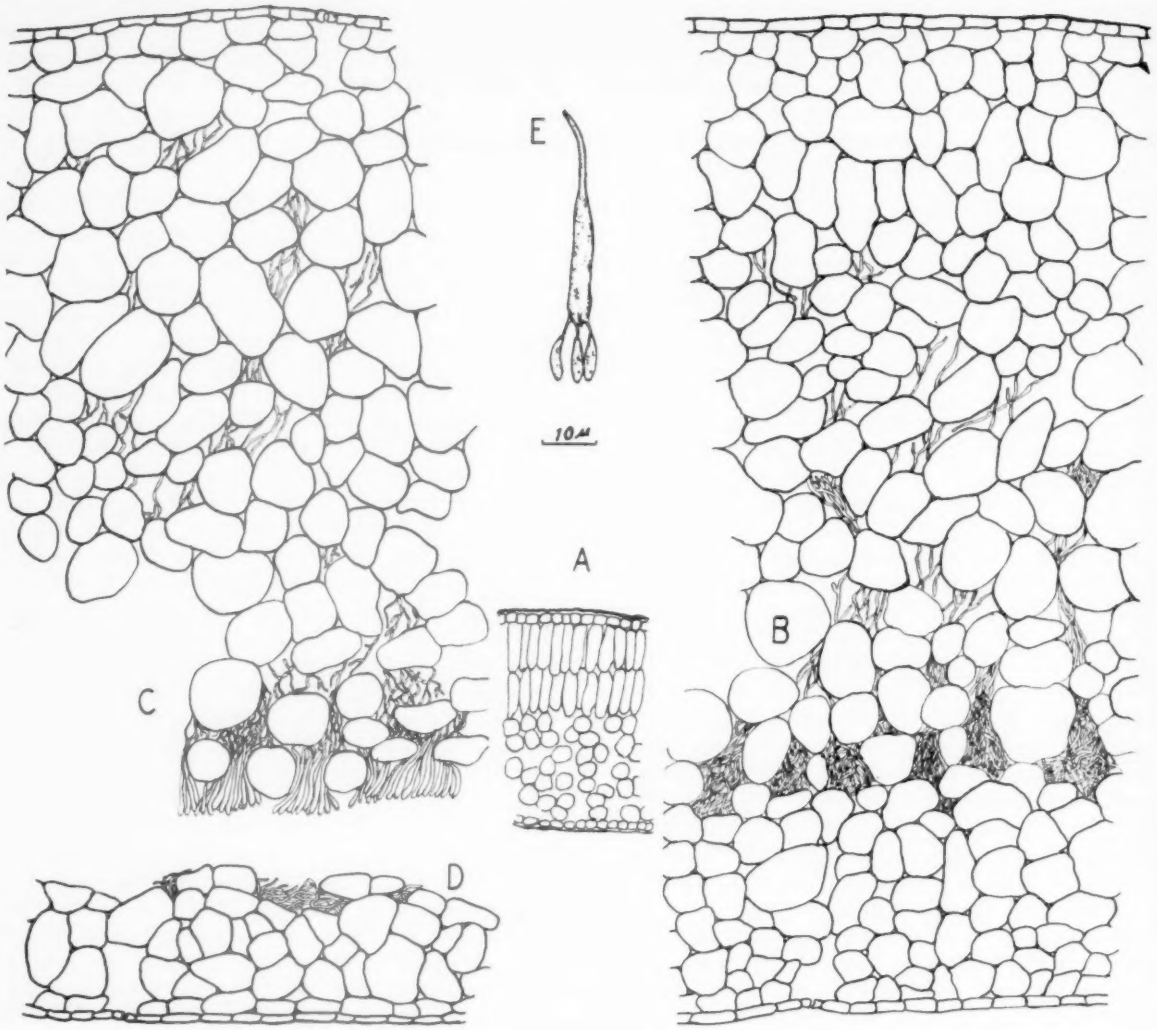

FIG. 1. Pathological changes in leaves of *Camellia sasanqua* induced by *Exobasidium camelliae* var. *gracilis*. A, B, C, and D drawn to the same scale; E, to scale shown. A, transverse section of normal leaf; B, transverse section of diseased leaf, showing hypertrophy and hyperplasia of the palisade cells and mesophyll, intercellular position of the pathogen, and early stage of hymenium formation within the leaf; C, diseased leaf from which the superposed membrane has been exfoliated by pressures produced by elongation of the basidia; D, section of the exfoliated tissues; E, basidium and basidiospores.

In seeking an explanation for the increase in size and number of leaf cells, histological features which characterize this disease, it was desired to determine whether *E. camelliae* var. *gracilis* has the capacity to produce indoleacetic acid. Accordingly, cultures of the organism were grown in each of two liquid media: Czapek's solution, containing 20 gm. sucrose, 3 gm. KNO_3, 1 gm. KH_2PO_4, 0.5 gm. KCl, and 0.5 gm. $MgSO_4.7H_2O$ per liter; and in a similar liquid medium prepared by substitution of 1 gm. per liter of *L*-tryptophane for potassium nitrate as the source of nitrogen.

Previously *E. camelliae* var. *gracilis* had been found to grow in a yeastlike manner in both liquid and solid media. In liquid media the cells are submerged at first, but after about 3 weeks a film forms at the surface.

The filtrates of 2-week-old cultures, grown in Czapek's medium and in the tryptophane-containing medium, were tested for the presence of indoleacetic acid by the ferric chloride-perchloric acid method of Gordon and Weber (3). Tests on the filtrate of cultures grown in Czapek's solution were negative, but those on the filtrates of cultures grown in the medium containing tryptophane were strongly positive, indicating that *E. camelliae* var. *gracilis* has the capacity to produce indoleacetic acid from tryptophane.

DISCUSSION.—It is presumed that the production of indoleacetic acid by *Exobasidium camelliae* var. *gracilis* at a time when the host tissues are embryonic

may account for both hypertrophy and hyperplasia of Camellia leaves. This presumption is supported by the results of previous studies of Levan (4) concerning the effect of indoleacetic acid upon onion root tips and of Friedman and Francis (2) with roots of tomato, which indicate that cell enlargement and abnormal stimulation of cell division are produced in these tissues by indoleacetic acid. Similar studies of other hypertrophic diseases might well reveal that indoleacetic acid is produced by the pathogens and is the proximate cause of the diseases.

The position of the hymenial layer with respect to the host tissues in *E. camelliae* var. *gracilis* differs from that reported in other species of the genus, the peculiar feature being that basidia begin their development within the leaf and become exposed when the lower epidermis and closely associated tissues are exfoliated. This is at variance with the observations on other species of Exobasidium, in which the hyphae extend between the epidermal cells and form basidia at the surface of the leaf. There seems to be nothing in the structure of the Camellia leaf that can be made to account for the formation of the hymenial layer internally. In fact, factors which may determine the position of reproductive structures among fungi generally, with respect to the substratum, remain largely unknown.

SUMMARY

Exobasidium camelliae var. *gracilis* causes a disease of *Camellia sasanqua* characterized by enlargement of all leaves on new shoots and exfoliation of the lower epidermis and contiguous tissues. Histological examination indicates that both hypertrophy and hyperplasia of host cells are involved. Differentiation of the leaf mesophyll is inhibited as a result of infection. Basidia begin their development within the leaf and cause a sloughing off of the lower epidermis and contiguous cells, whereupon the basidia are exposed and complete their development on the new surface resulting.

This organism in culture produces indoleacetic acid. It is suggested that this substance may account for a part of the pathological changes.

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HOST RANGE OF BOTTLEGOURD MOSAIC VIRUS AND ITS INACTIVATION BY PLANT EXTRACTS [1]

R. S. Vasudeva and T. K. Nariani

A mosaic disease of bottlegourd (*Lagenaria leucantha* (Duch.) Rusby) has been prevalent in the Delhi area for several years. The causal virus, designated Cucumis virus 2C by Vasudeva et al. (8), has a narrow host range and infects *Lagenaria leucantha*, *Cucumis sativus* L., and *Cucurbita moschata* Duchesne. It is characterized by its apparent inability to infect solanaceous plants, so that the problem has been to find out why the virus fails to do so.

INFECTION EXPERIMENTS.—Young solanaceous plants were raised, usually in 4-in. pots, in an insect-proof house in Delhi under normal conditions of light. When they had developed true leaves they were inoculated with sterilized cotton wool dipped in a standard extract of virus-infected bottlegourd plants. Carborundum powder had been added to the standard extract, which was prepared by adding 1 cc. of water to every gram of plant material, and the inoculated plants were kept under observation for over 4 weeks.

A variety of solanaceous plants, namely *Nicotiana tabacum* L., *N. tabacum* var. White burley, *Datura stramonium* L., *Solanum nigrum* L., *S. nodiflorum* Jacq., *Lycopersicon esculentum* Mill., and *Capsicum annuum* L., were inoculated by the usual method. Although under observation for more than a month, none of the plants showed obvious symptoms of the disease. In a second experiment of this type when plants had failed to develop symptoms of the disease 2 weeks after inoculation, subinoculations were made from each type of plant to bottlegourd to determine if the plants under test were carrying the virus. The results showed that the virus was carried in all symptomless plants tested except *Capsicum annuum* and *Lycopersicon esculentum*. Repeated inoculations from inoculated and noninoculated leaves both above and below showed that the virus does not move from

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inoculated leaves to noninoculated ones in any host plants which carry the infection. Movement of the virus in inoculated leaves was further tested in *Nicotiana tabacum* and *Datura stramonium* by the half-leaf inoculation technique. One half of a leaf was inoculated with bottlegourd mosaic sap after the other half was covered with cardboard, and inoculations were made on healthy bottlegourd after a fortnight from both inoculated and noninoculated leaf halves. The virus could be recovered from only the inoculated halves, which indicated that the virus does not move from the inoculation point. Virus could be recovered from inoculated plants of *Datura stramonium* and *Nicotiana tabacum* for as long as 46 and 35 days respectively after inoculation.

To find out whether the virus remained on the surface of inoculated leaves but failed to move into leaf tissues of the plants, six plants of bottlegourd and six of *Datura stramonium* were inoculated by placing infective sap on the leaf surface; and corresponding numbers of each host were inoculated by rubbing the sap onto the leaves with carborundum powder. Three plants in each lot were washed 10 min. after inoculation and the other three were not washed. Infectious sap placed on leaf surfaces was ineffective, because neither bottlegourds nor *Datura* became infected. Leaf abrasion with carborundum enabled the virus to enter leaf tissue of all the plants. Washing leaves 10 min. after inoculation did not alter the results secured by either inoculation method. Symptoms appeared on the six bottlegourds rubbed with the inoculum plus carborundum. It was necessary to inoculate bottlegourd with sap from the symptomless Datura leaves in order to prove presence of the virus in *Datura*. One or two of each set of three bottlegourd plants inoculated with sap from *Datura* became infected, which indicated that carborundum had enabled the virus to enter Datura tissues.

The nutrients in the sand in which Datura plants were grown were altered by irrigating the plants with full-strength nutrient solution[2] or 0.1 per cent potassium nitrate, to see if the virus would move beyond the point of inoculation in the plant. The Datura plants thus raised were inoculated with standard extract of bottlegourd mosaic virus and after a fortnight healthy bottlegourds were inoculated with sap from the inoculated and noninoculated Datura leaves. Sap from noninoculated Datura leaves failed to produce infection on bottlegourd, the evidence thus being that the virus had not moved from the inoculated leaves. Roots of *N. tabacum* plants were immersed in 2 per cent healthy bottlegourd leaf juice for 4–16 hr., and plants were later inoculated, but virus could not be recovered from noninoculated leaves of such plants.

EFFECT OF PLANT LEAF EXTRACTS ON THE VIRUS.—It was considered that the leaf extracts of solanaceous plants may adversely affect the infectivity of the bottlegourd virus. Juices of such plants as *N. tabacum*, *N. glutinosa*, and *Datura stramonium* prepared by grinding the leaves of healthy plants in a mortar were passed through sterilized cotton wool and added to standard extracts of bottlegourd mosaic virus in proportions needed to obtain virus concentrations of 1, 5, 10, 20, 40, 50, 60, 80, and 100 per cent. The extracts so prepared were intermittently shaken and kept for 1 hr. before they were used to inoculate healthy bottlegourd plants. In addition juices of *Cucumis sativus*, *Brassica campestris* var. *rapa*, *Spinacia oleracea* L., and *Cannabis sativa* L. were tested. Controls were of two types: the virus diluted with water and the virus diluted with juice of the normal host *Lagenaria leucantha*. Only the juice of *Datura stramonium* markedly inhibited the activity of the virus at 1 to 10 per cent virus concentrations, whereas the extracts of all the other plants tested did not inactivate the virus at the dilutions tested. Effect of the juice of *Datura stramonium* was repeatedly tested and results always were similar. Controls of both the types did not inhibit the virus.

In another experiment the effect of leaf extracts of *Datura stramonium*, *Nicotiana tabacum*, *Solanum nigrum*, *Lycopersicon esculentum*, and *Capsicum annuum* on the activity of the virus was similarly tested, as also was the effect of time of storage of mixtures prior to inoculation. The activity of the virus was inhibited when diluted 1:100 with the leaf juices of *Datura stramonium* and *Capsicum annuum* and when diluted 1:1000 with *Solanum nigrum* and *Lycopersicon esculentum*, whereas *Nicotiana tabacum* leaf juice did not affect the activity of the virus at either of these dilutions. The inhibitory action of *Datura stramonium* was effective also in a 1:10 virus-Datura juice mixture, but only after the mixture had been stored for 48 hr. before being used as inoculum. A similar effect was evident in a 1:10 virus-Capsicum mixture stored for 8 hr. A virus-toxic principle apparently is contributed to the mixture by such juices.

The leaf juices of *Datura* were studied further to determine if the toxic principle could be destroyed by heat or by desiccation. It was observed that Datura juice which had been heated to the boiling point did not adversely affect the activity of the virus even at a concentration of 5 per cent.

In another series of experiments the effect of desiccated Datura juice on the activity of the virus was tested. A known volume (10 cc.) of the leaf extract of *Datura stramonium* was dried for about 10 days over calcium chloride in a desiccator. The material so dried was then made up to original volume with sterile distilled water and was added to the standard extract of bottlegourd mosaic virus in different proportions. Bottlegourd was inoculated immediately and after storage for 1, 2, and 4 days. It was observed that the toxic principle is not adversely affected on desiccation and that the results of desiccated and fresh Datura juice at virus concentrations of 1:10 to 1:1000

[2] Nutrient solution: 0.25 gm. of $CaSO_4$, $Ca_3(PO_4)_2$ and $MgSo_4$; 0.08 gm. of NaCl; 0.70 gm. KNO_3; 0.005 gm. ferric chloride; 1000 cc. water.

were comparable whether inoculated immediately after dilution or after storage up to 4 days.

It was observed during these experiments that the inhibitory effect of Datura juices in different experiments was not always of the same order. It was therefore considered necessary to investigate the effect of juices of individual plants of *D. stramonium* of different ages. Plants 49 days and 113 days old were used. They were raised during October–December in the glasshouse and growth was poor, but the young plants had an average of three soft, light green leaves whereas the old plants had 10 thick, deep green leaves. The virus extracts diluted with leaf juices of young or of old plants were inoculated to young bottlegourd plants. Juices from young plants contained less of the toxic principle than those from old plants. Only when the inoculum mixture contained as little as 1 per cent virus extract did the juice from young Datura leaves inhibit virus activity sufficiently to prevent infection of two of the three bottlegourd plants. Juices from the old plants, however, prevented infection by the virus when the inoculum mixture contained up to 10 per cent virus extract. Whenever 20, 40, or 50 per cent of virus extract was in the inoculum mixture, the Datura juices failed to prevent infection of bottlegourd. Only one of the 18 control plants inoculated with the various water dilutions of virus extract failed to become infected. In another experiment the effect of dilution of the old Datura leaf juice prior to mixing with bottlegourd mosaic sap was tested, along with the effect of young and old Datura leaf juice. The results indicated that there is an appreciable fall in the inhibitory effect of the old Datura leaf juice when diluted 1:10 or 1:100 with water.

Discussion.—It has been shown that bottlegourd mosaic virus is carried symptomlessly in the inoculated leaves of *Datura stramonium*, *Nicotiana tabacum*, *N. tabacum* var. White burley, *Solanum nigrum*, and *S. nodiflorum*, but fails to infect *Lycopersicon esculentum* and *Capsicum annuum*. In *D. stramonium* and *N. tabacum* the virus remains confined to the inoculated portion of the leaves and does not move from the inoculation point.

Leaf extracts of *D. stramonium* and *Capsicum annuum* markedly inhibit the activity of the virus, and its infectivity is further affected by prolonged storage with these extracts. The greater the storage period, the more marked is their inhibitory effect. The extracts from old Datura plants show greater inhibition of the virus than do the extracts from young plants, and there is a fall in the toxicity of the leaf extracts when diluted. The fact that the inhibitory effect is lost on heating and dilution with water and is unaffected on desiccation indicates that some toxic principle is involved. The concentration of such a principle in the mixture determines the inhibitory property of the extract. Evidently the concentration of the toxic principle in young Datura plants is not high enough to inhibit the virus appreciably at the dilutions tested, and as the plants grow old, the toxic principle in the leaves markedly increases.

Duggar and Armstrong (2) reported the inactivation of tobacco mosaic virus by the juice of pokeweed (*Phytolacca decandra* L.) and *Datura*, while Elmer (3) showed that diluting the juice of mosaic-affected tobacco with filtered juices of healthy bean, cucumber, or tobacco plants reduced the infection by various degrees. Bawden and Kleczkowski (1) observed that the extract of strawberry plants reduced the infectivity of tobacco mosaic virus. Johnson (5) found that the extract of *Phytolacca rigida* Small almost completely inhibited the virus even after aging *in vitro* for long periods. Kuntz and Walker (7) have shown inhibition of tobacco mosaic virus and cabbage mosaic virus by extracts of spinach.

Grant (4), however, demonstrated that although sugar beet, Swiss chard, and spinach were susceptible to ordinary tobacco mosaic virus, the extracts from healthy plants of these species greatly reduced the infectivity of the virus. The inhibitory principle has been shown to be adversely affected by dilution as well as by heating. Johnson and Grant (6) have recorded irregularity in the amount of infection with spinach juice containing tobacco ringspot virus.

The findings recorded in this paper are of considerable interest mainly because the virus is carried in the inoculated leaves of certain Solanaceae and fails to infect others; also because leaf extracts of *Datura* and *Capsicum* markedly affect the activity of the virus. Further work is, however, required to determine the critical points at which the virus is being adversely affected by cell sap of different plants of the Solanaceae, particularly in relation to the period of interaction, as also to determine conditions under which the virus may extend farther from the inoculated parts of the plant.

SUMMARY

A mosaic virus of bottlegourd (*Lagenaria leucantha* (Duch.) Rusby) designated as Cucumis virus 2C infects inoculated but symptomless leaves of *Datura stramonium*, *Nicotiana tabacum*, *N. tabacum* var. White burley, *Solanum nigrum*, and *S. nodiflorum*. The infection is localized, being confined to the inoculated regions of the leaves. The virus fails to infect *Lycopersicon esculentum* and *Capsicum annuum*.

Leaf extracts of *Datura stramonium*, *Capsicum annuum*, *Solanum nigrum*, and *Lycopersicon esculentum* adversely affect the activity of the virus at different concentrations. In the first two, however, the activity of the virus is further affected by its prolonged storage with the extracts of these plants.

The toxic principle in Datura leaf extract is destroyed by heating it to the boiling point and is rendered ineffective on dilution; but it remains unaffected by desiccation.

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FUSARIUM ROOT ROT AND WILT OF DILL [1]

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Dill (*Anethum graveolens* L.) (3), although native to southeastern Europe, is now cultivated as a commercial crop in the United States as well as being grown in many home gardens. The seeds, flowering heads, and foliage are used as a condiment and in medicinal preparations (1). However, the main product from commercially grown dill is the essential oils which are used in perfumes.

In 1949 a very destructive wilt and root rot was discovered in some Ohio dill fields. The plants on one-fourth of one Ohio grower's acreage were completely destroyed by this disease, and the yield of oil from the remaining 200 acres was reduced an additional 25 per cent. The disease was equally destructive in certain fields in 1950.

A *Fusarium* (2, 7) was found to be the cause of this disease. Since no earlier report was found in the literature (4, 5, 6), a careful study of the disease and its causal organism was made. Selected results of the study are reported in this paper.

Symptoms.—*Seedlings.*—One of the first symptoms is a decreased growth rate of seedlings; frequently no other symptoms appear for several weeks. Usually the foliage changes from bright green to yellow, in the oldest leaves first. In some cases leaves become purple, and later yellow or rarely white. Occasionally the discolored leaves curl downward at the edges, but in most cases the entire plant wilts before any such curling becomes evident.

Many seedlings become infected and are killed before they emerge. Others emerge, grow rapidly until they are an inch or two in height, and then suddenly wilt and die; root systems of such plants were completely destroyed. When yellowing first appears most of the root system is still intact; however, the terminal portions of many of the fibrous roots usually have a dark orange to reddish-brown decay at this time. In more advanced stages of the disease all the secondary roots and most of the primary root and hypocotyl are destroyed. The decayed cortical tissues are dark brown; the vascular tissues are dark orange to reddish brown. Frequently there are dark lesions on the lower part of the hypocotyl of severely wilted seedlings. These lesions may be small and confined to one side of the hypocotyl, or may completely encircle it. If such plants are pulled, the hypocotyl usually breaks at or below the soil line leaving an orange to reddish-brown stump of vascular tissue surrounded by dark brown cortical tissues.

Older plants.—Dill plants of any age from seedlings to those in full bloom may suddenly wilt and die, but frequently no symptoms appear until after the plant has flowered.

The most striking symptoms in older plants are a change in leaf color from bright green to golden yellow, and the wilting of the entire plant which soon follows.

Yellowing appears first in the oldest leaves and extends upward into successively younger leaves. Usually the entire plant wilts and dies before the youngest leaves become yellow. In the older plants as well as in the seedlings, a purple coloration may immediately precede leaf yellowing.

The vascular tissues of the roots, crown, and lower stem of all wilted plants have an orange to reddish-brown or dark brown discoloration, extending from the point at which the fibrous roots are invaded up into the taproot, crown, and lower portion of the stem.

The amount and extent of root decay varies considerably from plant to plant. Most of the root system may be completely destroyed (Fig. 1); usually, however, there are many apparently healthy roots even on badly diseased plants.

Wilting is another characteristic symptom, often affecting the entire plant when infection is severe. During this study wilting preceded the death of all the plants; it may be sudden, or prolonged over 2 or 3 days, but once it has occurred the plant tissues gradually dry. Leaves on diseased plants become dry and brittle but do not abscise.

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FIG. 1. Root systems from dill plants: left, healthy; two on right, from plants infected with *Fusarium*.

About the time wilting occurs or shortly thereafter, a whitish growth of fruiting structures may appear on the surface of the stem just above the soil line. If conditions are favorable, pinkish buff-colored sporodochia are also produced on this region of the stem.

MATERIALS AND METHODS. — Certain methods and procedures that were followed throughout the investigations are described here. Variations from these occurred at times and will be discussed later. To isolate pure cultures of *Fusarium* from diseased dill plants, fragments of diseased tissue were placed on agar plates, and streak and spore dilution plates were made with conidia from the outer surface of diseased dill plants taken directly from the field. The pure cultures were established and maintained on 2 per cent potato-dextrose agar. Each culture was numbered, such as D4 for a mass isolate and D4-8 for a monoconidial isolate taken from D4. Both microconidia and macroconidia were removed with a glass needle from hanging agar drops.

For the pathogenicity tests the inoculum was increased on a steam-sterilized mixture of two parts oats and one part wheat. A 250-ml. portion of each culture was mixed with 1000 ml. of sterile soil. The soil was prepared by mixing one part of sand with two parts of clay loam and autoclaving the mixture in flats 3 in. deep for 2½ hr. at 18 lb. pressure.

In the greenhouse the soil crusted so badly that few seedlings emerged even in the check pots. To avoid this the seeds were covered with ½ in. of steam-sterilized sand.

Sterile clay pots were used for the greenhouse work. Each experiment was composed of four replicates and the pots were randomized when placed in the greenhouse.

All of the dill seed used in this study except a part of that used in resistance experiments came from one lot of seed. In all of the experiments the seed was sown immediately after the fungus cultures were added to the soil.

Since it was impractical to use all of the isolates, only those which were distinctly different from the others and those which were most pathogenic in preliminary tests were used. More than 90 per cent of the isolates had the same cultural and morphological characters. Therefore one of them, D4, was arbitrarily chosen to represent the group. Eighteen isolates could be separated on the basis of pigmentation, number and type of conidia produced, amount of aerial mycelium, etc.

RESULTS.—Infested soil was collected from each of three widely separated fields. The percentages of diseased plants were 90, 40, and 10 in fields 1, 2, and 3 respectively. Dill seed was sown in sterilized and nonsterilized portions of the soil from each field. Within 8 weeks the percentage of diseased plants in nonsterilized soil from the three fields was 100, 60, and 10, respectively. None of the plants that grew in the sterilized soil became diseased. A *Fusarium* was readily isolated from all the diseased plants.

Repeated attempts to isolate the *Fusarium* from dill seed obtained from many sources, including plants that had produced seed before they wilted, were unsuccessful.

Soil inoculation.—When separate pots of soil were inoculated with 19 isolates, all isolates were pathogenic. However, isolate D4 was considerably more pathogenic than any of the others. For example, 95 per cent of the seedlings failed to emerge from soil inoculated with isolate D4 whereas only 10–30 per cent failed to emerge from soils inoculated with the other isolates.

Monoconidial isolates were used in other pathogenicity and virulence studies. In one set of tests with 11 monoconidial isolates, six came from isolate D4 and five were taken directly from a diseased plant stem, D23. Two in each group were microconidial isolates, the others macroconidial. To increase the percentage emergence from soil inoculated with the

TABLE 1.—*Pathogenicity to dill of 11 monoconidial Fusarium isolates when cultured on a wheat-oats medium, and mixed with sterilized soil*

Isolate No.	Percentage emergence	Condition of plants 60 days after planting		
		Percentage:		
		Diseased	Dead	Healthy
Check	73	0	0	100
D4	65	0	99	1
Micro[a]				
D4-0	65	4	87	9
D4-2	72	9	82	9
D23-0	74	9	85	6
D23-1	71	17	63	20
Macro[a]				
D4-8	70	1	99	0
D4-9	76	0	100	0
D4-10	66	0	100	0
D4-11	68	2	98	0
D23-6	69	8	91	1
D23-7	69	1	98	1
D23-8	72	3	96	1

[a] Microconidial and macroconidial isolates.

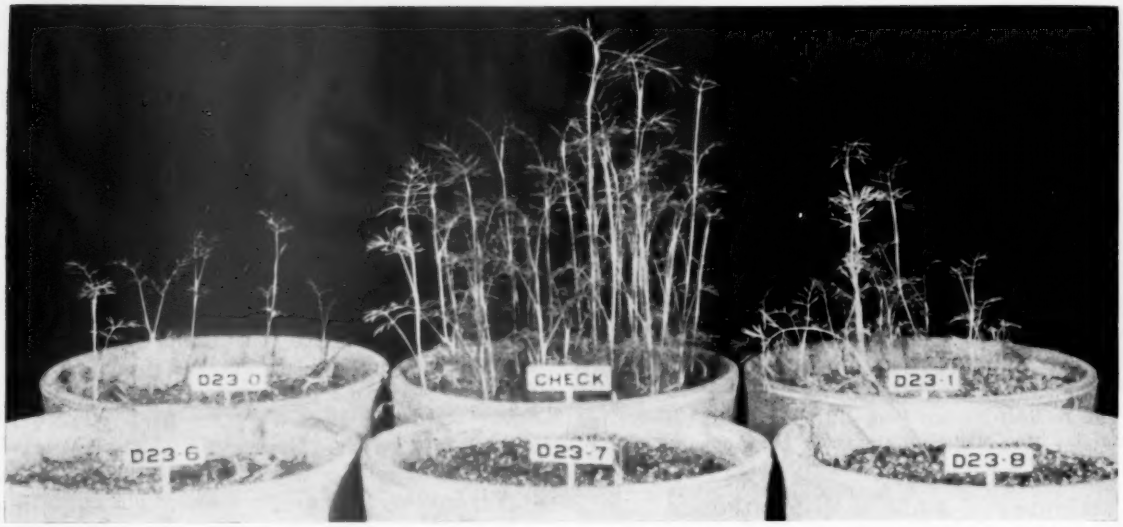

FIG. 2. Dill plants 8 weeks old, in soil inoculated with monoconidial isolates made directly from the stems of diseased dill plants.

highly virulent isolate D4 so that symptoms could be more readily studied and compared. ¼ in. of sterile soil was placed between the inoculated soil and the seed. All the monoconidial isolates were pathogenic to dill (Table 1). However, the seven macroconidial isolates were highly virulent, 99 or 100 per cent of the plants being dead or diseased after 60 days. The four microconidial isolates were somewhat less virulent, especially isolate D23-1. This difference in virulence is shown in figure 2.

When the inoculum was produced on a cornmeal medium the pathogenicity of the various isolates was essentially the same as on the wheat-oats mixture. Furthermore, each of the monoconidial isolates became established in the soil and infected seedlings when only a water suspension of spores from the isolate was added to the soil.

In another experiment a mixture of spores and mycelial fragments was washed into the soil around plants of different ages. Again there were apparent differences in virulence of the different isolates, but the virulence of all the isolates decreased as the age of the plants at the time of inoculation increased. In general the youngest plants were the most susceptible.

Reisolation.—At least six dill plants per isolate were chosen at random from those which became diseased while growing in inoculated soil. Plants were selected for each of the monoconidial isolates and for each mass isolate that was distinctly different from the others. The monoconidial isolates were indistinguishable from each other on the basis of cultural and morphological characters.

In making the reisolations, pieces of infected tissue were cut from each plant and surface-sterilized for 3 min. in a 1:1 solution of Clorox and 95 per cent grain alcohol. The tissue fragments were transferred directly from this solution to agar plates. Cultures of the fungus were obtained from each plant on both water agar and 2 per cent potato-dextrose agar. The reisolated fungi were culturally and morphologically the same as the original cultures placed in the soil.

Platings were made from sections of the roots, crown, and several different stem regions of plants that wilted after attaining a height of 12 in. or more. Many of the plants were in flower when the yellowing and wilting first appeared. In all of these plants Fusarium cultures were obtained from the roots, crown, and lower stem, but the fungus was never obtained from tissues more than 2 in. above the crown. The *Fusarium* always grew most luxuriantly from crown tissue fragments. Cultures from the crown and lower stem tissues were almost always pure. Colonies of bacteria and other fungi were frequent, along with those of *Fusarium*, in platings made from badly infected roots.

Host range.—Among the plant species tested for susceptibility to this pathogen (listed below), some were closely related to dill, the others being varieties of crop plants that have been reported as being susceptible to certain species of *Fusarium* and a few varieties reported to be resistant to *Fusarium*. Four varieties of cabbage were used because cabbage was included in the crop rotation where this disease was first found. The plants and varieties used were: celery, Giant Pascal, Dwarf Golden Self Blanching; parsnip; parsley; carrot, Red Cored, Ox Heart; beet, Early Wonder, Detroit Dark Red; cabbage, Yellows Resistant Wisconsin Hollander, Danish Ball Head, Copenhagen Market, Early Jersey Wakefield; muskmelon, Rocky Ford, Honey Rock, Pride of Wisconsin; watermelon, Cole's Early, Kleckey's Sweet; spinach, New Zealand, Nobel; squash, Zuccini, Straight Neck; tomato, Rutgers, Stokesdale, Ponderosa, Earliana.

Seeds were obtained from The Capitol Seed Com-

pany, Columbus, Ohio, and planted in a bed that was 6 in. deep containing a mixture of the soils used in some of the previously described experiments. Thus the soil in which these plants grew contained several isolates of the fungus. Rows of dill seed were planted at intervals throughout the entire bed.

Dill plants in all parts of the bed became diseased and the *Fusarium* was readily isolated from these plants.

In the first trial only a few seedlings of some of the varieties emerged. Therefore, a second planting of each of these varieties was made in infested soil in pots. This time the emergence of all the varieties was over 85 per cent and there was no significant difference in percentage emergence between the checks and those planted in the infested soil. None of the varieties used in these trials is susceptible to the various isolates of the Fusarium.

Dill seeds were sown also in soil known to be naturally infested with *Fusarium conglutinans* Wr. and in soil inoculated with the same organism isolated from diseased cabbage plants. None of the dill plants in either of these two soils became diseased. Therefore, the *Fusarium* that parasitizes dill is different from the Fusaria which are pathogenic to cabbage and the other crop varieties tested.

Seed Treatment.—Dill seed was treated with Yellow Cuprocide (90 per cent cuprous oxide), Spergon (96 per cent tetrachloro-para-benzoquinone), Semesan (30 per cent hydroxy-mercurichlorophenol), Arasan (50 per cent thiram), New Improved Ceresan (5 per cent ethyl mercury phosphate), and Ceresan M (7.7 per cent ethyl mercury p-toluene sulfonanilide) at the rates recommended for seeds of comparable size. The treated seeds were planted in pots of soil that was inoculated on the day of planting. Rate of emergence was highest from seed treated with Cuprocide, but the plants which emerged soon became diseased. Rate of emergence was considerably reduced by both New Improved Ceresan and Ceresan M. However, in the plants which did emerge from seed treated with New Improved Ceresan, symptoms did not develop until after they flowered. Arasan, Semesan, and Spergon had no apparent effect upon the seedlings or the fungus.

Since New Improved Ceresan appeared to protect the seedlings from invasion long enough to be of commercial importance, its effect was retested by planting treated dill seed in soil in which the fungus was previously well established. In this experiment New Improved Ceresan failed to control the disease.

Resistance.—None of the large seed companies could provide seed of a variety of dill other than the one planted in Ohio. Apparently only the one variety is cultivated throughout the world.

Another attempt was made to locate wilt resistance in plants from a field that had been abandoned because more than 90 per cent of the plants had been killed by this fungus. Seed from 40 plants free of symptoms but growing close to diseased plants was planted in infested soil, and more than 99 per cent of the resultant plants became diseased. Seed from the different seed companies was also planted in infested soil. At the present time no resistant selections have been made. However, since certain plants remain healthy while growing among others that become diseased, apparently some individual plants have a certain degree of resistance.

SUMMARY

A destructive root rot and wilt of dill (*Anethum graveolens* L.) was first discovered in Ohio in 1949. A new *Fusarium*, isolated from the diseased plants, proved pathogenic to dill plants growing in soil inoculated with pure cultures of both single-spore and mass isolates of the fungus.

The symptoms of the disease include browning and rotting of the roots, brown discoloration and necrosis of the vascular system, yellowing of the foliage, and wilting which is followed by death of the plant.

Dill plants of all ages are susceptible; those between 1 and 2 weeks old are the most susceptible.

The pathogen is confined to the tissues of the lower stem and roots of living dill plants.

Physiologic races of the fungus were found.

In experiments on host range, no infection was obtained on several varieties of peas, parsley, parsnips, celery, carrots, beets, cabbage, tomatoes, squash, muskmelon, watermelon, and spinach.

A few dill plants growing close to diseased plants in infested soil remained healthy, but most of their progeny proved to be highly susceptible.

Arasan, Ceresan M, Cuprocide, New Improved Ceresan, Semesan, and Spergon failed to give satisfactory control of the disease when used as seed treatments.

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MUTATION IN A TOBACCO NECROSIS VIRUS STRAIN [1]

Robert W. Fulton

The tobacco necrosis disease was shown by Bawden (1) to be caused by several serologically unrelated viruses which produced similar symptoms on tobacco and bean. It also has become evident that strains of at least some of these viruses exist naturally (2, 4, 5) or may be selected from mixtures (3).

Six strains were described previously from this laboratory (3) on the basis of symptom differences on cowpea (*Vigna sinensis* Endl. var. Blackeye). Five of these consistently reproduced characteristic lesions during many transfers. The sixth, strain A, normally produced lesions which varied from concentric white rings to nonringed white spots, depending on environment. After several successive transfers, using as inoculum many lesions rather than one, some distinct red lesions also were produced. Repeated observation indicated that the red-lesion types were mutants of the white-lesion strain. The occurrence of these mutations is described here.

MATERIALS AND METHODS.—Numerous hosts were tried, but none was found as suitable as cowpea for distinguishing between different strains. Some strains indistinguishable on tobacco and other hosts produced distinctly different symptoms on cowpea; consequently cowpeas were used almost exclusively. The plants were inoculated, using carborundum, when the primary leaves were about three-fourths expanded, and immediately rinsed with water. By pinching off the trifoliate leaves as they developed, abcission of the infected primary leaves was prevented for 2 weeks or longer.

After inoculation the plants were placed 4 to 6 in. below three 40-watt daylight fluorescent lights. The plants were exposed to these lights for 12 hr. a day and were screened from daylight. The temperature was maintained at about 80° F. Under these relatively constant conditions strain A produced lesions consisting of concentric white rings. These are referred to simply as white lesions, while the term red lesion refers to any type of lesion which consisted mainly or entirely of red necrotic tissue.

Most inoculations were made from single lesions or portions of lesions ground in 0.1 to 0.5 ml. of pH 7 phosphate buffer. Lesions were punched from infected leaves with sterile cork-borers or small sharpened metal tubes.

RESULTS.—When cowpeas were inoculated with a single white lesion ground in buffer, only white lesions appeared after 4 days. Later, as the lesions increased in size, small red necrotic areas or spots appeared at the margins of some of them. These usually enlarged and became wedge-shaped (Fig. 1, A), resembling sectors in a fungus colony. Sometimes the white lesion developed more rapidly than the red spot, and eventually completely surrounded it (Fig. 1, B).

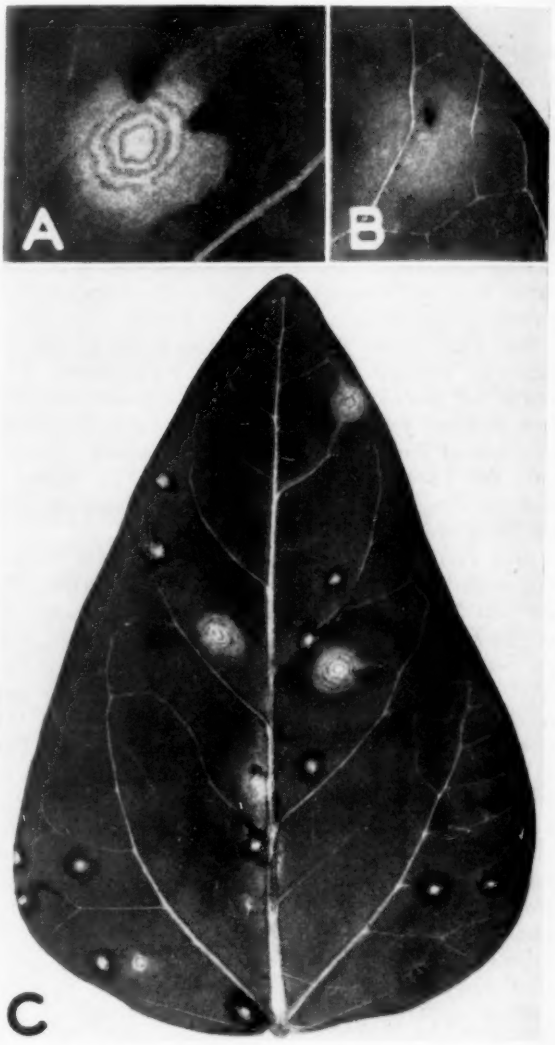

FIG. 1. A (× 6) and B (× 3.5), red necrotic spots appearing at the edges of normally white lesions of tobacco necrosis virus strains A and A1 on cowpea. C, a cowpea leaf inoculated with red necrotic spots similar to those shown in A and B, and showing well separated red and white lesions.

Transfers were made by punching the red areas from the leaves, including as little as possible of the white tissue. On subinoculation to healthy cowpeas, well separated red and white lesions were produced (Fig. 1, C). Transfers from the original lesions, using portions which did not contain red necrotic tissue, produced only white lesions on cowpea.

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A number of lines of evidence indicated that the red sectors were the result of mutation and were not contaminants in the original inoculum. The red necrosis first appeared only within, or at the margin of, a white lesion. Well separated red lesions were produced, however, from inoculum containing a mixture of both red and white necrotic tissue. On leaves inoculated with known mixtures the red lesions appeared 1 to 2 days before the white lesions. This further indicates that this mixture in the inoculum was readily separable.

Red necrosis did not invariably indicate the origin of a mutant strain. Normally white lesions showed some reddening under lower light intensities or when temperatures varied 5° to 10° above or below 80° F. This type of red necrosis usually was diffuse and involved entire lesions or the entire margin. Transfers from such lesions produced only white lesions on cowpea leaves. Red necrosis due to a change in the virus, on the other hand, appeared as sharply limited spots.

Since tobacco necrosis virus strain A produced mutants rather frequently, an attempt was made to determine whether other similar isolates behaved in the same way. Naturally infected greenhouse-grown tobacco roots provided inoculum which was obviously a mixture of types. From this inoculum 11 single lesion strains were selected which produced white lesions on cowpea. Mutants causing red necrotic sectors appeared on lesions produced by each of these isolates.

Some of the isolates produced fewer mutations than others. Two which seemed to represent extremes were compared with strain A in a number of trials. Lesions of strain A1 were smaller than those of strain A and showed only a slight tendency to form rings. Lesions of strain A2 were similar to those of strain A. Inoculum for each trial consisted of a single white lesion which showed no red necrotic sectors. The white lesions and any red mutants were counted 7 days after inoculation. The results are in table 1. Strain A1 consistently produced the most numerous mutations, strain A2 producing the fewest, except in one trial.

The trials in table 1 were run consecutively, inoculum consisting of a single lesion without mutants selected from the preceding trial. In spite of this selection there appeared to have been little or no elimination of the tendency to mutate.

TABLE 1.—*Relative frequencies of mutation from white-lesion type to red-lesion types in three isolates of tobacco necrosis virus on cowpea*

Trial	Number of mutants per 10 white lesions for isolates [a]		
	A	A1	A2
I	1.28	2.10	0.0
II	0.83	1.24	0.0
III	0.66	3.10	0.98
IV	1.57	4.47	0.81
V	1.23	3.85	0.75
VI	1.25	3.65	0.0
VII	1.00	2.33	0.48
VIII	1.29	5.31	0.15
Ave.	1.14	3.26	0.63

[a] Total lesions for isolate A = 775; isolate A1 = 1348; isolate A2 = 603.

Similar trials were run in an attempt to select for the tendency to mutate by transferring white portions of lesions which already showed two or more red mutants. Eight to 10 serial transfers from such lesions failed to change the rate at which mutants appeared.

While some of the isolated mutant strains appeared similar, many varied in the symptoms produced on cowpea. Types were obtained which closely resembled the previously described (3) strains C, D, E, and F. No isolate was found, however, which resembled strain B. Some isolates were obtained which differed from those previously described, chiefly in producing concentric red rings.

The range of variation of mutants from one parent strain seemed to be about the same as from another. The red necrotic sectors appearing in lesions of strain A1 were usually smaller than with the other two strains. The red-lesion strains obtained from them, however, showed no consistent differences on comparisons with mutants of strains A or A2.

DISCUSSION.—The origin of a variety of strains of a tobacco necrosis virus from a single "stem" strain indicates the potentialities of this strain. The sudden appearance of strains in nature, particularly adapted to certain hosts, might thus occur because of the presence of a relatively innocuous but mutable strain. Strain A or types very similar to it have been found in most naturally infected tobacco roots examined, indicating that strain A is able to compete successfully with its mutants on this host. On other hosts, however, it might be rapidly overgrown by mutants causing a more severe or fatal disease.

The differences in numbers of mutants produced by strains A, A1, and A2 seem to indicate variation between the strains not correlated with symptom type. For several reasons the figures given do not represent mutation rates. The relative rates of multiplication of the three strains may not be the same. Some evidence indicated that strain A1 either multiplied more rapidly than the other two or was more infectious. Another possibility is that the appearance of a mutant may depend to some extent on its ability to compete with the parent strain and multiply sufficiently to produce a red necrotic sector. The apparent stability of these differences between white-lesion strains may be due partly at least to the difficulty in recognizing and selecting more or less mutable types.

The strains producing red lesions, although not investigated extensively, showed no tendency to mutate. While a mutant causing red necrosis might be difficult to detect if its parent caused only a slightly different type of red necrosis, mutations back to the white type should easily be seen. These apparently did not occur. Here too the possibility exists that on cowpea the red lesion types dominate in competition with the white lesion types. This would account for the rela-

tive frequency of the white-to-red mutation and the apparent absence of reverse mutations.

Because these red-lesion strains resemble mutations in microorganisms, in their apparently spontaneous and random appearance, the term mutant has been applied. It may be that virus mutations are not comparable to mutations in more complex forms. On the other hand, it is possible that a study of mutations in viruses, presumably composed of a high proportion of "genetic" material, might be of value in determining the nature of such changes in higher organisms. The strain of tobacco necrosis virus used here seems to possess certain advantages in that it gives rise to distinctly different strains which remain local and are readily observed and isolated.

SUMMARY

Red necrotic spots appeared at the margins of slowly spreading white lesions on cowpea leaves inoculated with a single lesion strain of a tobacco necrosis virus. These spots resembled sectors, and first appeared only in association with the white lesions. From them were obtained, on subinoculation, strains producing only red necrotic lesions. Leaves inoculated with a mixture of red and white lesions showed well separated red and white lesions, the former appearing sooner than the white lesions. For these reasons it was concluded that the red spots were mutants.

All of 11 isolates causing white lesions on cowpea produced red mutants. Of three strains studied in detail, the most unstable strain produced mutants about five times as frequently as the most stable strain. Selective transfers from lesions showing mutants did not increase the numbers of mutants produced by the subcultures. Many of the red mutants differed from each other in the type of lesions produced on cowpea. There were no apparent reverse mutations from red to white lesion types.

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SOME RESPONSES OF GRAPEVINES TO SODIUM ARSENITE SPRAY APPLIED FOR BLACK MEASLES CONTROL [1]

Wm. B. Hewitt

Black measles—an odd but economically important disease of *Vitis vinifera* L.—is characterized by black spotting of the skin of the berries along with a form of interveinal leaf burn. The symptoms may appear on only a few canes or over the entire vine; they may develop rather suddenly, and may show one season and not the next. Black measles may be partially controlled by spraying dormant vines with sodium arsenite. Experiments have been carried out in California for the past ten years to study the relationship between spraying of vines and control.

The few to many fruit spots are usually small, round, and characteristically bordered by a halo and a ring of dark tissue. In severe cases the entire berry may be affected. Such measled berries often crack, wither, and dry up on the vine. Symptoms are more conspicuous on white or light-colored grapes than on the berries of dark-colored varieties.

Leaf symptoms usually develop on canes which have diseased fruit, occasionally on canes on which the fruit is apparently normal. The interveinal browning and drying of the leaf tissue, typical of black measles, starts as small, yellowish spots between the primary veins that gradually enlarge and become necrotic in the center. Leaves of colored varieties of grapes develop red margins around the burned necrotic areas. In extreme cases only narrow bands of green tissue remain along the five large leaf veins.

Vines on which sudden and very severe symptoms of black measles appear usually drop most of their leaves and the canes die back from the tips. This form of the disease has been called apoplexy (4). Such vines often recover and symptoms may not appear again the next year.

The cause of black measles is not certain. Bioletti (1) in 1923 combined several vine troubles into this general group and attributed them to mismanagement. In 1926, Viala (4) reported that *Sterium necator* Viala caused the disease by invading the functioning vascular elements from decaying wood in the trunks. In California, the disease has been found on vines which have not had any form of trunk rot.

Black measles has been controlled in Europe and in California by a dormant application of sodium arsenite solution. This control was found among experiments to control Pyralid moth, as reported by

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Viala (4), and was introduced into California in 1926 by Bonnet (2). The function of sodium arsenite in the control of black measles is not understood. An answer to this question probably awaits an understanding of the cause or causes of the disease.

Bioletti (1) stated that vines may show symptoms of black measles one season and appear normal the next. Recommendations for control (2) were to treat all vines when large numbers were diseased, but only diseased vines when there were but a few.

SPOT TREATING COMPARED WITH SPRAYING ALL VINES.—The question of spot treating as compared to spraying all the vines in a vineyard was studied. Replications were laid out in alternate blocks in an Emperor vineyard and all vines showing symptoms were marked. Diseased vines in four plots were treated by swabbing the trunks and arms with sodium arsenite solution; in the four alternate plots all vines were sprayed. Vines in two border plots were held as controls and were not treated. The results of these tests as observed the next season are in table 1.

TABLE 1.—*Percentage of Emperor vines with black measles before and after spot treatment of only diseased vines, as compared with spraying all vines*

Replication	Hand-treated; diseased vines only Percentage		Spray-treated; all vines Percentage	
	Before treatment	After treatment	Before treatment	After treatment
1	23	35	13	5
2	29	21	23	9
3	13	13	26	6
4	15	6	19	13
Control	10	14	11	21

Spot treating, or the application of sodium arsenite to the trunks and arms of diseased vines only, reduced the percentage of measled vines in only one of four plots, whereas spraying all vines in the plots reduced the diseased vines an average of nearly 60 per cent in three of the four plots and 30 per cent in the fourth as compared to the previous season. The percentage of measled vines increased in both control plots. In some cases, vines which did not show disease the season before treatment were diseased after treatment.

EFFECT OF DIFFERENT CONCENTRATIONS OF ARSENITE ON CONTROL.—An experiment to study the effects of different concentrations of arsenite on the control of black measles and the shift of vines from diseased to normal, and vice versa, was laid out in a Thompson Seedless vineyard. The 2835 vines were classified and mapped in August 1943 into healthy, H, or one of five symptom classes from A to E in order of severity. Four treatments were replicated, each three times, in randomized blocks of about 235 vines each. The treatments were no-spray and dormant spray applications of three different concentrations of sodium arsenite solution. Expressed as arsenic trioxide, the spray concentrations were: 1.5, 2.5 (recommended concentration for black measles control), and 3.5 lb. per 100 gal. of water. In August 1944 all vines were again classified, and each vine was compared with its previous season's history.

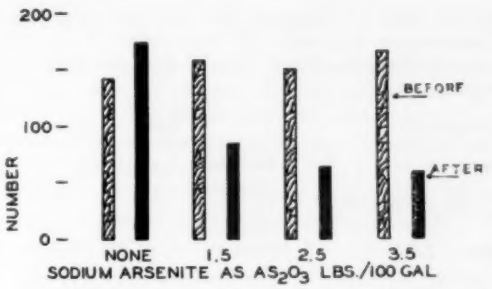

FIG. 1. The number of diseased vines in nonsprayed plots and in sprayed plots before and after a dormant-season spray application of three different concentrations of sodium arsenite.

Analysis of variance of the data from the replicated plots in four treatments showed that spraying grapevines for black measles with sodium arsenite was an effective control, for all sprayed plots were significantly healthier than the nonsprayed at odds greater than 99:1, but none of the sprayed plots was better than any other. Even though no one of the three spray concentrations of sodium arsenite was significantly better than the others, there was a decrease in the number of diseased vines in the combined totals of the replications for each increase in arsenic concentration (Fig. 1). From 1943 before spraying to 1944 after spraying there was a 22 per cent increase in the diseased vines in the nonsprayed plots and a decrease of 47, 58, and 64 per cent in diseased vines with each respective increase of arsenic concentration.

SHIFTING OF VINES FROM HEALTHY TO DISEASED AND VICE VERSA.—Of the 555 healthy vines in the unsprayed plots in 1943, 452 remained healthy and 103 became diseased; or, there were 18.5 per cent new cases of black measles (Table 2); in the plots sprayed with sodium arsenite concentrations of 1.5, 2.5, and 3.5 (expressed as lb. As_2O_3 per 100 gal. of water) there were respectively only 8.0, 7.1, and 6.7 per cent new cases of disease. There were 147 diseased vines in the unsprayed plots in 1943 and 76 or 51.6 per cent of them recovered in 1944. In the dormant spray concentrations 1.5, 2.5, and 3.5, the percentage recovery was respectively 76.7, 85.3, and 86.5. This greater recovery and also the fewer new cases of black measles in the sprayed over unsprayed plots indicated that the sodium arsenite served as a therapeutic agent in the control of black measles.

Vines shifted irregularly from one symptom class to another in all plots, sprayed and nonsprayed. Table 3 illustrates the distribution of the number of vines which were healthy and the number in each symptom class, both before spraying in 1943 and again after spraying in 1944, and the shift of vines from one symptom class to another. Under the heading not treated, there were 555 healthy vines in 1943, and in 1944 only

TABLE 2.—*Shift of Thompson Seedless vines from healthy to diseased and the reverse after no treatment and after dormant-season spraying with three different concentrations of sodium arsenite solution*

Treatment	1943		1944		Recovered (per cent)	New cases (per cent)
			Healthy	Diseased		
Unsprayed	Healthy	555	452	103		18.5
	Diseased	147	76	71	51.6	
Sprayed, 1.5 lb.[a]	Healthy	561	516	45		8.0
	Diseased	160	122	38	76.7	
Sprayed, 2.5 lb.	Healthy	554	515	39		7.1
	Diseased	150	128	22	85.3	
Sprayed, 3.5 lb.	Healthy	538	502	36		6.7
	Diseased	171	148	23	86.5	

[a] Dormant spray concentrations of sodium arsenite are expressed as lb. of As_2O_3 per 100 gal. of water.

452 of these same vines remained healthy, 23 of them shifted to class A, 21 to B, 48 to C, 9 to D, and 2 to the very severe class E. Of the 52 vines in the disease class C in 1943, 28 shifted to healthy in 1944 even though they were not sprayed. The remainder shifted into classes as follows: 8 to A, 1 to B, 10 to C, 4 to D, and 1 to E. Three of the 8 vines which were classified as very severe in 1943 were healthy in 1944, and only 2 of the vines remained in their original symptom class.

In the sprayed plot of Table 3, 554 of the 704 vines in the three replications were healthy in 1943 and 515 of them remained healthy in 1944 after spraying, while 22 of them shifted to symptom class A, 6 to B, 10 to C, and 1 to D. Similarly, of the 64 vines classed in B in 1943, 58 shifted to healthy after spraying, 1 to A, 2 to B, 2 to C, and 1 to D.

TABLE 3.—*Distribution of Thompson Seedless vines in symptom classes in plots with no treatment and plots receiving a dormant spray of sodium arsenite*

Number of vines in 1943 symptom classes		Number of vines in 1944 symptom classes					
		Healthy (H)	Diseased				
			A	B	C	D	E
Not treated							
Healthy (H):	555	452	23	21	48	9	2
Diseased:							
A	9	4	1	1	2	1	
B	55	32	3	6	10	3	1
C	52	28	8	1	10	4	1
D	23	9		1	6	5	2
E	8	3			1	2	2
Total vines	702	528	35	30	77	24	8
Sprayed[a]							
Healthy (H):	554	515	22	6	10	1	
Diseased:							
A	13	11	1	1			
B	64	58	1	2	2	1	
C	53	44	2	1	5		1
D	14	10	1	1	2		
E	6	5		1			
Total vines	704	643	27	12	19	2	1

[a] Dormant spray concentration 2.5 lb. sodium arsenite as As_2O_3 per 100 gal. water.

The failure to obtain satisfactory control by spot-treating diseased vines only (Table 1) may in part be explained by the shift of vines from healthy to diseased. For example, in the nontreated plots (Table 2) 103 of the 555 healthy vines in 1943 shifted to diseased classes in 1944. Had the 150 originally diseased vines in this plot been spot-treated, there would have been at least 103 new cases of disease in addition to the old ones that would not have been cured. In the plots sprayed with arsenite concentrations of 1.5, 2.5, and 3.5, the number of vines which shifted from healthy in 1943 to diseased in 1944 after spraying was only 45, 39, and 36, respectively. It could be expected, then, that the shift from healthy to diseased would be greater when diseased vines only are treated than when all vines are treated.

SPRAYING BEFORE OR AFTER PRUNING.—Because it is necessary to prune grapevines early and spray late in the dormant season to avoid excessive arsenite injury through pruning and leaf-scar wounds (3), the feasibility of spraying before pruning to control black measles was compared with spraying after pruning. Since the commercial application of spray to only one side of the vine would materially reduce the cost of treatment over spraying both sides, the two methods were also compared. Randomized plots were laid out in an Emperor vineyard. Sodium arsenite spray at a concentration of 2.5 lb. (expressed as As_2O_3) per 100 gal. water was used. The treatments were: no-spray, sprayed on one side of the vine, and sprayed on both sides of the vine. Both spray treatments were applied to a series of plots before pruning and to another after pruning. The results of these tests (Table 4) show that when compared with no treatment, the spraying

TABLE 4.—*Percentage of Emperor vines with black measles after spraying with sodium arsenite only one side or two sides of the vine, before and after pruning*

Treatment time	Number of vines—percentage diseased		
	Control (no treatment)	Sprayed	
		One side	Two-sides
Before pruning	891—10.66	445—8.76	444—5.85*
After pruning	834—13.19	419—5.73*	419—3.34*

* Significant at 99:1 odds.

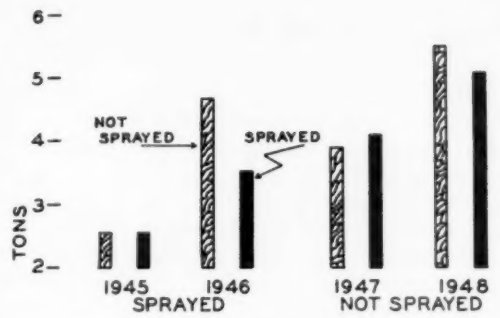

FIG. 2. The effect of repeated dormant sprays with sodium arsenite on the yield of Gamay vines, sprayed in 1945 and 1946 and unsprayed in 1947 and 1948. The irregular yield from season to season of the unsprayed plots is characteristic of the past history of this particular vineyard.

of vines on one side only before pruning did not significantly reduce the disease incidence, but spraying both sides of the vine reduced the incidence from 10.6 to 5.8 per cent. Spraying the vines after pruning gave significant reductions in measled vines, from 13.2 to 5.7 per cent in the plots sprayed only on one side, and 3.3 per cent in plots sprayed on both sides.

EFFECTS OF SUCCESSIVE ANNUAL SPRAYS. — Some growers have sprayed their vineyards two and even three successive seasons with sodium arsenite in an effort to cure all measled vines. Experiments to determine the effects of this practice have been completed to date in three varieties of grapes. Sprayed and nonsprayed plots of 120 vines in a vineyard of the variety Gamay were each replicated seven times. Sprays were applied in January 1945 and again about the same time in 1946, but were not applied in 1947 or 1948. The second application of sodium arsenite reduced the yield in 1946 about 20 per cent when compared with the nonsprayed plots (Fig. 2). The sprayed plots recovered their yield the next year and were not significantly different from the nonsprayed plots in 1947 and 1948.

Vines of the variety Muscat Canelli sprayed one, two, and even three successive seasons with the concentration of sodium arsenite recommended for the control of black measles (2.5 lb. As_2O_3 per 100 gal. of water) did not show any significant differences in yield. Thompson Seedless vines receiving the same treatments did not show any deleterious effects of the arsenite spray on yield.

It appears then that successive annual applications of sodium arsenite solution will reduce the yield in some varieties of *Vitis vinifera*, such as Gamay, but that repeated applications to varieties like Thompson Seedless may not affect yield. Thus it appears that it will be necessary to test each variety separately for tolerance to sodium arsenite spray.

SUMMARY

Spot-treating only the black-measled vines in a vineyard with sodium arsenite solution during the dormant season did not reduce the incidence of disease, but spraying all vines reduced the disease by an average of over 50 per cent.

A dormant spray of sodium arsenite on Thompson Seedless vines at concentrations (expressed as lb. of As_2O_3 per 100 gal. water) of 1.5, 2.5, and 3.5 significantly reduced the number of measled vines when compared with nonsprayed. The difference between any two of the three sprays was not significant, but the totals of the three replications in each spray treatment showed a decrease of 47, 58, and 64 per cent in diseased vines with each increase in arsenite concentration. There was an increase of 22 per cent measled vines in the nonsprayed plots.

New cases of black measles increased 18.5 per cent in unsprayed plots and only 8.0, 7.1, and 6.7 per cent in plots sprayed with 1.5, 2.5, and 3.5 concentrations of arsenite respectively. There was 51.6 per cent recovery of diseased vines in the unsprayed plots and 76.7, 85.3, and 86.5 per cent recovery in the sprayed plots.

Thompson Seedless vines classified into healthy and five symptom classes in order of severity in the seasons before and after spraying with different concentrations of sodium arsenite were shown to shift from one symptom class to another. The shift of vines was irregular and occurred in both sprayed and nonsprayed plots, but was greater from diseased to healthy in the sprayed, and the opposite in the nonsprayed.

Spraying entire vines gave better control than spraying only one side, and spraying after pruning gave better control than spraying before.

Two successive annual sprays of sodium arsenite reduced the yield 23 per cent in the Gamay variety after the second application. Similar treatments to Muscat Canelli and Thompson Seedless had no effect on yield even after three annual sprays.

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STUDIES ON THE DISEASE CYCLE OF BLACK ROT OF CRUCIFERS [1]

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While the black rot disease, incited by *Xanthomonas campestris* (Pam.) Dowson, has received much study since it was first described on rutabaga by Pammel (14) in 1895, certain phases in the disease cycle need further investigation. These have to do with 1) details of penetration of natural openings and of roots by the pathogen, 2) the manifestation of symptoms in relation to temperature, and 3) the nature of seed contamination and infection. Results of a study of the first two phases are presented in this paper.

EXPERIMENTAL RESULTS.—*Stomatal penetration.*—Hydathode infection of cabbage leaves by the black rot pathogen was discovered by Smith (19) and confirmed by Russell (17). Neither of these investigators observed penetration through stomata. Invasion of the unspecialized stomata in the sinus of cabbage cotyledons was described by Drechsler (7). Clayton's (4) report of penetration through the stomata on the lower surface of uninjured leaves of cauliflower and cabbage is the only one in which stomatal invasion of true leaves in nature has been recorded. These observations have not been confirmed and the present writers have never observed evidence of stomatal penetration in true leaves of cauliflower and cabbage in the field. Infection through the hydathodes was regarded by Meier (12) to occur only when a liquid path of entry for the pathogen was present. By adding a surface tension depressant (sodium oleate) and a sticker (gelatin) to the inoculum, Bhide (1) obtained stomatal infection in young cabbage plants.

In repeated attempts by the writers to induce penetration and infection by way of stomata in cabbage and cauliflower, young succulent plants were sprayed with suspensions of the pathogen and the plants were kept in a moist chamber for 2 days. Except in two cauliflower leaves, in which stomatal invasion possibly occurred, only the typical marginal lesions resulting from penetration of hydathodes appeared. Since this procedure failed to induce penetration of stomata it was postulated that no continuous layer of liquid existed between the infection drop and the substomatal chamber, and that such a layer might be essential for penetration. Accordingly attempts were made to encourage invasion by inducing water congestion in substomatal chambers.

Seeds of cabbage (var. Jersey Queen) and cauliflower (var. Early Snowball) were sown in flats of a sand-soil mixture kept at 20° C. Plants were transplanted into individual pots 4 weeks later. After another 4 weeks the roots of these plants were subjected to artificial water pressure by use of the apparatus described by Johnson (11). Water congestion of cabbage leaves was always apparent first in the vicinity of the hydathodes. With continued treatment signs appeared inwardly ½ in. or more along the leaf surface. A few small, scattered spots of water-soaked tissue appeared also in the center of most leaves. Visible congestion did not last for more than 6 hr. after treatment unless the plants were placed in a moist chamber immediately after treatment. No adverse reaction followed recession of congestion. In cauliflower plants treated in a similar manner the response was less marked and was slower.

When the maximum degree of congestion was obtained the plants were atomized with a suspension of the bacteria in distilled water, care being taken to avoid injury by holding the atomizer at least 12 in. from the leaf. Inoculated plants were then held in a moist chamber for 24 hr. before they were returned to the greenhouse bench. When signs of the disease appeared, parts of affected leaves were fixed in formalin-aceto-alcohol and dehydrated by the dioxan-butyl-alcohol method (18). All collected material was embedded in paraffin (54°–56° C.), and sections were cut 8μ in thickness with a rotary hand microtome. Sections were affixed to the slide with Haupt's affixative (10) and were stained by the Triarch Quadruple Stain technique (6).

First indications of stomatal infection appeared on cabbage plants about 4 days after inoculation, about the same time that hydathode lesions became visible: small, slightly sunken, dark spots, always on the lower leaf surface, usually remaining less than 1 mm. in diameter (Fig. 1, A). Sometimes an extensive area along the midrib became black from the coalescence of smaller lesions. Although cauliflower leaves did not show signs of stomatal infection quite so early as did those of cabbage, similar lesions were apparent after 6 days, usually remaining small; coalescence was not observed. Hydathode lesions were more numerous on cauliflower leaves than on cabbage leaves.

Cabbage and cauliflower plants, not artificially water-congested, were also inoculated by atomizing inoculum on both leaf surfaces. With cabbage, no stomatal infections were ever found, but with cauliflower two lesions on two plants of approximately 50 inoculated appeared to result from stomatal invasion.

Examination of slides prepared from leaves with stomatal lesions revealed that the substomatal chambers on the lower leaf surfaces often contained large numbers of bacteria (Fig. 1, B). Further invasion was confined largely to the intercellular spaces of the spongy mesophyll, but occasionally the pathogen became established in the intercellular spaces of the palisade tissues. When lesions developed so extensively, the affected portion of the leaf was always considerably more sunken than the surrounding healthy

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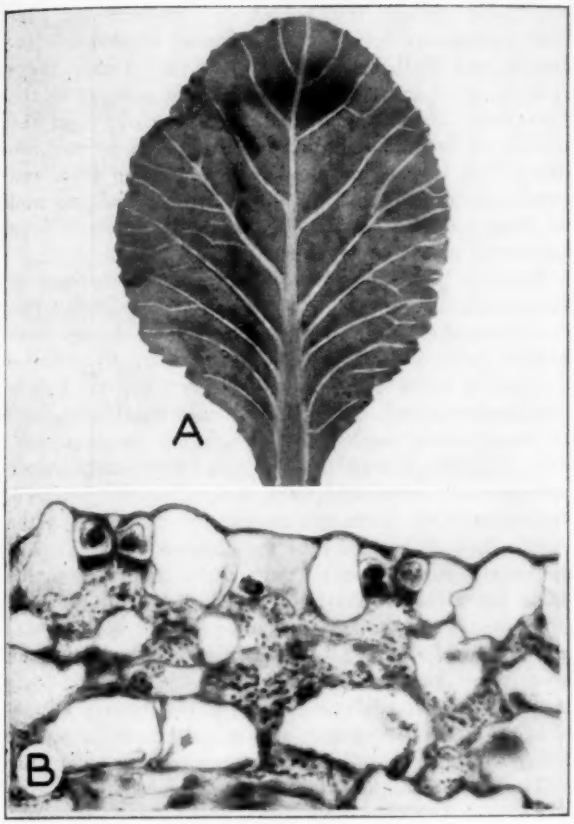

FIG. 1. A, cabbage leaf showing stomatal infections on lower leaf surface 4 days after inoculation with *Xanthomonas campestris* following water congestion by subjecting the roots of the plant to artificial water pressure. Plant placed in moist chamber for 24 hr. following inoculation. Approx. 2×. B, photomicrograph of a portion of the lower epidermis and spongy mesophyll cells from cabbage leaf shown in A. Bacteria can be seen in substomatal cavities and intercellular spaces as well as quite near the stomatal opening, indicating stomatal penetration. Approx. 1200×.

tissue, because of the collapse of the mesophyll cells involved. Regardless of the degree of invasion, however, no infection of vascular tissue was found.

It would appear from these studies that water congestion in natural openings of cabbage and cauliflower is necessary for penetration by the black rot organism. Subjection of the roots of young cabbage and cauliflower plants to water under artificial pressure results in water congestion in some substomatal cavities and intercellular spaces of the lower side of the leaf. Water may then serve as a path of entry for the pathogen. In nature, congestion in these hosts is confined to the epithem-hydathodes. It is induced in the stomata on the lower sides of the leaves only by the application of relatively high pressures through the roots. When congestion is produced, penetration of stomata takes place as readily and symptoms develop as rapidly as in hydathodes. These facts are interpreted as showing that absence of stomatal invasion of cabbage and cauliflower leaves in nature is not owing to the glaucous nature of the leaf surface, nor to the structure of the stomata. Rather, they suggest that water congestion in the natural openings is necessary for penetration, and stomatal invasion is absent in nature because water congestion seldom occurs in substomatal chambers.

Root penetration.—Russell (17) stated that the black rot pathogen never infects cabbage plants through the root system. Smith (20) also thought that roots were not invaded, but Potter (15) and Middleton (13) were of the opinion that the bacteria may gain entrance into plants through the roots. Stewart and Harding (22) proposed infection by way of the roots and Harding, Stewart, and Prucha (9) believed that invasion through underground organs was not infrequent. Soil inoculations by Smith (21) failed to produce diseased plants even though the root system was injured. Drechsler (8) found no evidence of root infection and assumed invasion in his experiments to occur through the stomata in the sinus of cotyledons. Brown and Harvey (2) concluded from field observations that infection of Chinese cabbage plants was through the stem, the roots, or the bases of leaves. Cauliflower plants that had symptoms 3 weeks to a month after transplanting were thought by Clayton (5) to have been infected through the cotyledons while in the seedbed. Richardson (16) concluded that the pathogen may invade rutabaga seedlings from the soil.

Eight-weeks-old Jersey Queen cabbage plants, each in an individual 4-in. pot, were inoculated by one of two methods: 1) 50 ml. of inoculum was poured over the soil in each pot, the plant having been undisturbed since transplanting (4 weeks previously); 2) 50 ml. of inoculum was poured over the soil in each pot after the plant had been removed from the soil, the roots clipped with shears, and the plant replaced in its original pot without watering. All inoculum was prepared by suspending the bacterial growth from a 6-day 6-oz.-bottle slant culture in 100 ml. of distilled water. Following inoculation, the soil in each pot was covered to a depth of ½ in. with a fresh sand-soil mixture. In each experiment several noninoculated plants were placed adjacent to those inoculated, and an air temperature of 20°–24° C. was maintained. A cumulative record of all symptoms expressed by each plant was obtained.

In most experiments, some leaves on the plants inoculated after the roots were clipped showed a type of reaction, previously unassociated with this disease, as early as 13 days after inoculation (Table 1). Small, faint, chlorotic spots could be seen in parts of the affected leaves when viewed from above. These spots were not visible, however, by transmitted light. The affected portions of the leaf varied considerably in size and in location on the lamina. Quite often a whole leaf was densely spotted, but occasionally only one-fourth or less of the total surface of the lamina, usually an area near the leaf base, was affected. Leaves

TABLE 1.—*Number and type of leaf lesions resulting from inoculation of (A) uninjured[a] and (B) clipped[b] roots of cabbage seedlings*

Days after inoculation	Chlorotic spotting		Marginal lesions		Systemic lesions	
	A	B	A	B	A	B
13	0	40	0	0	0	23
18	12	60	1	2	8	60
22	19	79	5	8	22	129
26	20	85	5	10	39	183
31	22	88	5	11	56	235
35	22	90	7	12	62	264
39	22	90	8	12	66	287

[a] One hundred plants inoculated.
[b] Sixty plants inoculated.

which had this spotting were decidedly more numerous on those plants whose roots were injured before inoculation. On about 85 per cent of the plants which had atypical spots as the first symptom, typical marginal (Fig. 2, A) and systemic leaf lesions (Fig. 2, B) appeared later. The pathogen was never reisolated from the atypical spots. The symptom is thought to be the result of a toxic product of the organism which diffused through the tissue in advance of the organism.

Eighteen days after inoculation typical marginal and systemic symptoms were found on plants whose roots were inoculated by both procedures. Of 100 plants inoculated through uninjured roots, 13 developed symptoms and seven of these died during the course of the experiments. Of 60 plants whose roots were injured at the time of inoculation, 44 developed symptoms and 15 of these died. Plants whose roots had been clipped had more leaves with systemic lesions than plants not so treated. This relation continued throughout the experiments, there being on an average, about three times as many lesions at the conclusion of the study on the plants whose roots had been clipped as on those whose roots were not disturbed (Table 1). Leaves with marginal lesions were markedly fewer in number than leaves with systemic lesions, but as with the systemic lesions the marginal lesions were most numerous on plants whose roots had been injured at the time of inoculation.

The pattern of symptom expression.—Black rot is a common epidemic disease on cabbage in Wisconsin, where sudden widespread outbreaks after midseason have been shown to be traceable to primary infection in the seedbed. As pointed out by Walker (23) in the analysis of such an epidemic in the northeastern part of the state in 1940, early symptoms on cotyledons or lower leaves of seedlings disappear with the dropping of those parts and external symptoms are often absent for several weeks while the pathogen apparently develops systemically. With increasing summer temperatures external lesions eventually appear, often at the leaf margins, and a new crop of inoculum is available for secondary spread under favorable environment. While Burkholder (3) pointed out that many waterpore lesions are the result of systemic infection and Walker (24) illustrated such a case, there is little or no exact information on the subject in the literature. Since this is an important phase of the disease cycle it was studied by the writers. Some evidence that typical marginal lesions develop from systemic infection of the leaf through the petiole as well as from invasion of the hydathode has already been presented in the previous section.

Cabbage seedlings (var. Jersey Queen) grown in the greenhouse at about 20° C. were inoculated when 6 or 8 weeks old; two of the lowermost leaves were rubbed with a glass spatula dipped in a bacterial suspension after the leaves had been dusted lightly with carborundum. Following inoculation, plants were divided equally among the following air temperatures: 16°, 20°, 24°, and 28° C. Noninoculated plants placed adjacent to inoculated ones never became infected, indicating that no marginal invasion occurred in the relatively low humidity of the greenhouse.

Disease development commenced in some plants with the atypical chlorotic spotting of one or more noninoculated leaves per plant. This symptom was similar to that already described in the section on root penetration and is regarded as the result of toxic materials which diffused through the tissues in advance of the pathogen, since all attempts to isolate the pathogen from spotted leaves were unsuccessful.

The next symptom of the disease was expressed on

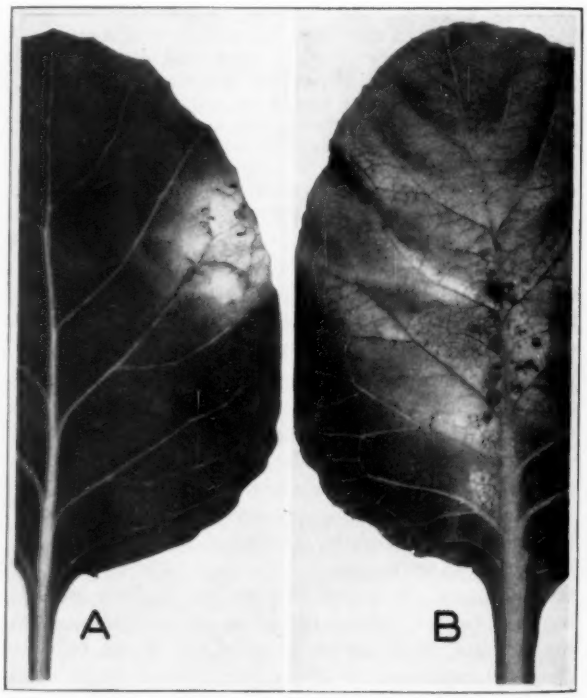

FIG. 2. A, marginal lesion which appeared 23 days after inoculation with *Xanthomonas campestris* on plant held at 20° C. B, leaf with lesion which appeared first near the midrib 28 days after inoculation on plant held at 24° C.

TABLE 2.—*Number of leaves with chlorotic, marginal, and midrib symptoms in 8-weeks-old cabbage plants inoculated and held at the air temperatures indicated*[a]

Days after inoculation	Chlorotic spotting				Marginal lesions				Midrib lesions			
	16°	20°	24°	28°	16°	20°	24°	28°	16°	20°	24°	28°
11	0	0	0	0	0	3	3	2	0	0	0	0
19	0	0	0	6	1	17	17	8	0	0	0	2
27	0	0	0	8	3	23	17	8	0	6	5	5
35	0	2	0	8	3	23	17	8	0	12	19	25
43		2	0	8	3	23	17	8	0	17	25	46

[a] Forty plants were inoculated at each temperature; all noninoculated check plants remained healthy.

noninoculated leaves as marginal lesions identical in appearance with lesions resulting from hydathode infections (Fig. 2, A). This symptom began to appear 11 days after inoculation and continued to be the predominant symptom type at all air temperatures for another 8 to 12 days on plants 8 weeks old at inoculation. On plants 2 weeks younger when inoculated, marginal lesions were always observed first at the three higher temperatures. Symptoms consisted almost entirely of marginal lesions on the younger plants at 16°, although such lesions did not appear until about 19 days after inoculation. Such symptoms were slightly more common at 20° and 24° in plants which were 8 weeks old at inoculation. The veins situated within leaf areas affected by these lesions were always visibly darkened and the pathogen was frequently isolated from such regions. Leaves having these symptoms usually dropped within 10 days.

Soon after the appearance of marginal lesions, another type of lesion was observed, always apparent first at some point along the midrib, but eventually involving a considerable portion of the lamina. The veins in the tissues affected were consistently darkened (Fig. 2, B). On plants 8 weeks old at inoculation, the midrib symptom became predominant at the three higher temperatures approximately 27 days after inoculation, but was not found on the plants at 16°. With the younger plants this symptom was progressively later in appearing with a decrease in air temperature, the first midrib lesions occurring 15 days after inoculation at 28° but only after 31 days at 16°. In general there was also a progressive increase in total number of lesioned leaves from 16° to 28° in plants of both age groups. The results of a typical experiment with 8-weeks-old plants are in table 2.

To determine whether marginal lesions may appear as a result of systemic invasion, a series of tissue transfer isolations was made from 150 individual leaves, each of which had a marginal lesion, collected from plants inoculated only on a lower leaf. In the laboratory the collected leaves were placed for 20 sec. in $HgCl_2$ (1:1000) and rinsed in sterile, distilled water. Three to six tissue transfers were made from each leaf, depending upon its size. The first transfer was always a section from the inner margin of the lesioned area and each subsequent transfer was made approximately 1 in. inward from the preceding one. This procedure was continued along the vein nearest the lesion to its junction with the midrib and thence down the midrib to the base of the petiole. The last transfer was made from the base of the petiole. All petiole sections were sliced into small pieces so as to permit more cut surfaces of the isolated tissue to be in contact with the medium. All isolated tissues were placed immediately on sterile nutrient-dextrose-agar plates and incubated at room temperature for at least 4 days before results were recorded. From the 150 leaves assayed by the tissue transfer isolation technique the organism was recovered from nearly all of the successive fragments in 53. In 27 leaves recovery was made in each fragment from the marginal lesion to the base of the petiole. In 21 leaves the organism grew out from each fragment except that taken from the base of the petiole. In five cases recovery was made from each fragment but one, the latter being from an intermediate location between the leaf margin and base of the petiole.

DISCUSSION.—It has been definitely established that the black rot pathogen may gain entrance to the plant through the hydathodes, but only when these structures are filled with guttation fluid. That stomata are relatively unimportant in infection, whereas hydathodes are the major portals of entry, cannot be explained on a structural basis since stomata and hydathodes are quite similar in gross morphology. Since these structures are so similar morphologically and the causal organism is a motile bacterium having one polar flagellum, ruling out motility as a prerequisite for stomatal penetration, some other character must distinguish the two structures as to relative susceptibility and immunity. Both stomata and epithem-hydathodes are involved in the water relations of the plant, but they do not function in the same manner. The guard cells of stomata, by opening and closing the stomatal aperture, regulate to a large extent the rate of loss of water as water vapor from the plant. The guard cells of hydathodes are stationary and, therefore, cannot modify the hydathodal opening. Because of a vein termination at each epithem there is a constant supply of water in the vicinity of the hydathodes. This results in a filling of the intercellular spaces of the

epithem, and, under conducive conditions, a liquid exudation (guttation) from the hydrathodes. Upon recession of conditions favoring such hydathode "congestion," the exuded water remaining at the hydathodes is drawn back into the plant by capillary action. Suspended microorganisms thus gain entrance into the plant in the vicinity of vascular tissue. Although there is usually water in the form of water vapor in the substomatal cavities, natural condensation is apparently not sufficient to supply a continuous liquid path of entry for the causal bacteria. Stomatal invasion can then occur only when the suscept has been subjected to unusual conditions which induce a filling of the substomatal chambers with water. The limiting factor for both hydathode and stomatal invasion is, therefore, one and the same, i.e., a continuous liquid path of entry.

Although inoculum was sprayed on both leaf surfaces, and there are stomata on both surfaces of cabbage leaves, there seems to be no reason why some infections should not have occurred through stomata of the upper leaf surfaces unless such infection was prevented by the lack of natural or induced water congestion. The veins in cabbage leaves are more closely associated with the spongy mesophyll tissues than with the palisade layers. Diffusion of transpiration water, then, is largely through these less compact mesophyllous cells in the direction of the substomatal cavities of the lower leaf surfaces. This is also the probable path taken by the excess water introduced into the plant by subjecting the roots to water under pressure. Thus, the stomata on the lower leaf surfaces would more likely be congested, and, in all probability, the stomata of the upper leaf surfaces did not become congested sufficiently to provide a liquid path of entry for the pathogen.

Considerable conjecture has been offered concerning infection through the root system, but little supporting experimental evidence has been given. No opinions have been advanced as to the reason for such invasion not being plausible, probably owing to the accepted fact that most bacterial pathogens do not generally directly penetrate healthy, uninjured epidermal tissues. The path of invasion into those uninjured plants which became diseased from contaminated soil in these experiments was not determined. However, since the incitant most probably may exist in the soil, for a time at least, penetration of the underground organs may well result from this close association if roots are injured. In roots of young growing plants, injury to the cortical and epidermal tissues and exposure of these tissues in close proximity to the xylem commonly accompany the origin of secondary roots. The soil used in these experiments was not examined microscopically, but in all likelihood microfauna were present that could have caused external injuries to the roots of the experimental plants. Also, during transplanting the vascular tissue of the root system is unavoidably exposed, rendering young plants susceptible to invasion by the black rot pathogen if the soil is so contaminated.

The path of entry by the pathogen, following inoculation by the rubbing method, was not determined, but it is suspected that infection was through wounds produced on the leaf. In these experiments conditions were unfavorable to hydathode infection, and no marginal lesions were observed on inoculated leaves or on leaves of adjacent, noninoculated plants. However, typical marginal lesions resulted from inoculation of two of the lowermost leaves without any intervening systemic lesions. The marginal lesions reported are therefore regarded as resulting not from hydathode invasions but rather from systemic infection. Apparently the bacteria reached the termination of the xylem elements where they lodged in the epithem of the epithem-hydathodes and multiplied sufficiently to induce lesion formation. Disease development in young cabbage plants usually proceeds so rapidly under favorable environmental conditions that it is impossible to detect variations in symptom expression. However, if the introduced inoculum is not sufficient to induce rapid killing, as was the case with the rubbing inoculation, and the environment is not so favorable, external evidences of disease conform to a definite pattern for a time. With an increase in air temperature from 16° to at least 28° C., the causal bacterium is progressively favored, while the environment for the suscept becomes increasingly less favorable above 20°. Coincidentally with a progressive decrease in air temperature below 28°, multiplication of the introduced bacteria is slower, as is disease development, until at 16° very few symptoms appear. The rates of increase and of transfer of the bacterial population are influenced so greatly by air temperature that the predominant type of symptom expressed by diseased plants may thus be completely reversed within a range of 12°. Because of the slower increase of the pathogen at the lowest temperature, marginal lesions were later in appearing than at the higher temperatures. Because invasion by the pathogen of a single element in the vascular system of a plant is sufficient to induce a marginal lesion, isolation by tissue transfer was not expected to establish definitely the presence or absence of the bacteria in each fragment. A single bacterium or small aggregation of bacterial cells could well reach the termination of an element and produce a marginal lesion unaccompanied by previous uninterrupted invasion by the organism throughout the vessel.

The lesions which originated along the midrib are thought to result from plugging by the bacteria of the smaller xylem elements in the secondary veins in the lamina. This is made possible by the rapid increase of the introduced inoculum in the larger xylem elements of the midrib. The elements in veins of the laminae are usually considerably smaller in diameter than those in the midrib. In one instance this difference was as much as 10μ, and several of the smaller elements were only 12μ in diameter. Plug-

ging by the bacteria of vessels with a larger diameter would not be hard to visualize in view of the fact that the pathogen produces a copious matrix in culture. At the lowest temperature multiplication of the bacteria was apparently not sufficiently rapid to induce such plugging, and marginal lesions predominated over those near the midrib.

SUMMARY

In nature stomata are unimportant in relation to penetration by the black rot pathogen, since entrance of the bacterium is dependent upon a continuous path of liquid in the stomatal aperture and the substomatal chamber. By subjection of the roots of young cabbage and cauliflower plants to relatively high artificial water pressure before the leaves were atomized with a bacterial suspension, water congestion and subsequent infection through stomata on the lower leaf surfaces were obtained.

It was demonstrated that mechanical injury to the roots of cabbage seedlings was not a prerequisite for infection by way of the underground organs. However, a much higher percentage of infected plants was obtained when mechanical injury to the roots immediately preceded inoculation.

Disease development in mildly affected young cabbage plants, 6 and 8 weeks old at inoculation, was studied at 16°, 20°, 24°, and 28° C. Symptom expression conformed to a definite pattern as concerns two symptom types. For the first 19 days after inoculation, characteristic marginal lesions predominated at the three higher temperatures. From this time until 35 days after inoculation there was a rapid transition in type of symptom expressed until systemic lesions originating in more central areas of the leaves were markedly predominant. At the lowest temperature, nearly all observed symptoms were marginal lesions. A third symptom type, a chlorotic spotting not previously associated with black rot, often appeared soon after inoculation and was attributed to diffusion of a toxic substance through the tissues in advance of the causal organism itself. From tissue transfers made from leaves with marginal lesions, it was determined that in these experiments both marginal lesions and those in the internal areas of the leaf lamina were the result of systemic infection rather than of invasion of the hydathodes.

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STUDIES ON HELIXIN IN RELATION TO PLANT DISEASE CONTROL [1]

Curt Leben and G. W. Keitt [2]

In a recent paper (6) from this laboratory, studies on the production, partial purification, properties, and *in vitro* activity of a fungitoxic antibiotic named helixin were reported. The present paper is concerned with further investigation of this material, especially in relation to plant disease control. It deals with the effect of pH on the activity of the partly purified antibiotic and with its fungitoxic and phytotoxic properties. A brief report on part of the work has been previously published (5).

MATERIALS AND METHODS—*Antibiotic preparations.*—Two partly purified helixin preparations made as indicated previously (6) were used for these studies. Both were obtained from shaken flask fermentations: 1, containing 2.5 units per mg., was used for the leaf washing and stability tests; and the other, containing 2.7 units per mg., was used for the remainder of the experiments. Ethanol was the solvent for both preparations. In studies using plants, the ethanol was removed by vacuum distillation over water and the water preparation[3] applied to the plant. In the agar-streak tests the ethanol preparation was diluted with water to the extent that the ethanol had no inhibitory effects on test organisms as indicated by suitable controls.

Agar-streak tests.—Agar-streak tests were made as described previously (3), using inoculum derived from potato-glucose agar (6) cultures incubated from 4 to 7 days at 22–24°C. The antibiotic was added in varying amounts to potato agar (6), which was adjusted with sterile NaOH or H_2SO_4 to the desired pH level after autoclaving and before pouring the plates. Plates were streaked with the test organisms and incubated for 4 days at 24° before data were recorded.

Early blight foliage tests.—The protectant action of the antibiotic was studied in greenhouse tests with early blight disease of tomato, incited by *Alternaria solani* (E. and M.) Jones and Grout. The methods of McCallan and Wellman (7) as modified in earlier work (4) were used to produce the disease. Dosage-response curves were plotted on the basis of the percentage disease control afforded by 4 concentrations of the antibiotic, 3 plants being used for each concentration in each trial.

Leaf disk assay.—A leaf disk assay patterned after that described for antimycin (4) was used in studies of stability and washoff. The assay depends upon the diffusion of antibiotic from treated leaf disks into an agar medium seeded with an indicator fungus. After a period of incubation there is formed around the disk a zone of inhibition related in size to the amount of antibiotic on the disk. This amount, expressed in arbitrary "leaf units," is determined by reference to a dosage-response curve drawn on the basis of the size of inhibition zones produced by known amounts of helixin carried in paper disks. Details of the assay were the same as for the antimycin leaf disk assay (4) except that: 1) *Glomerella cingulata* (Stoneman) Spauld. and v. Schrenk was used as the indicator; 2) the medium and method of seeding were the same as for antimycin (1); and 3) filter paper disks[4] carrying the standard ethanol preparations were dried before using. Disks dried after being dipped in aqueous preparations at pH 4.0, 5.7, and 7.7 produced similar dosage-response curves; it appeared unlikely, therefore, that the pH of preparations used for spraying leaves influenced leaf disk assay results.

RESULTS—*Effect of pH on antibiotic activity.*—In the first few protectant studies with early blight, several nearly flat dosage-response curves were obtained. Examination of spray preparations used in these tests revealed that higher concentrations were more acidic than lower concentrations, indicating that the antibiotic was less active under acidic conditions. To examine this point, agar-streak tests were made, using media adjusted to different pH levels. Results of these tests are given in Table 1. These experiments demonstrated that the antibiotic was indeed less effective under acid conditions. This finding was confirmed in early blight protectant tests, described below.

Studies of helixin as a protectant toxicant—(a) *The dosage-response curve.*—The protectant action of a partly purified preparation of helixin was determined in 5 series of experiments with the early blight test. The pH of spray solutions was adjusted to 5.7–6.0 with NaOH. The average LD50 value was found to be 6.5 μg. per ml. (range: 0.9 to 14); the average LD90, 25.5 μg. per ml. (range: 5.3 to 38); and the average LD95, 37.9 μg. per ml. (range: 9.5 to 60). The slopes, based on average determinations, were: LD50/LD90 = 0.18, LD50/LD95 = 0.25. The LD value range in these tests was great; this could perhaps be attributed to the fact that tests were made at different times of the year under conditions that could not be closely controlled.

The influence of the pH of the antibiotic spray on the level of early blight disease control was examined

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[2] Assistant Professor and Professor, respectively. The authors wish to thank G. J. Stessel for his assistance in various phases of the work.

[3] The antibiotic was precipitated from water solutions more acid than pH 4-5 (6). These preparations were applied as suspensions.

[4] Schleicher and Schuell No. 740-E.

TABLE 1.—*The influence of the pH of the medium on the inhibition of various organisms by helixin. Agar-streak tests*[a]

Organism	Inhibition point[b] at: pH 4.7	pH 5.8	pH 7.4
	μg. per ml.	*μg. per ml.*	*μg. per ml.*
Alternaria solani (E. and M.) Jones and Grout	1.4	1.4	<0.7
Candida albicans (Robin) Berkhout	11.3	11.3	2.8; 1.4
C. zeylanoides (Cast.) Langeron et Guerra[c]	11.3	5.6	2.8
Glomerella cingulata (Stoneman) Spauld. and v. Schrenk	5.6	1.4	1.4
Hansenula saturnus (Klöcker) Sydow[c]	11.3	11.3	2.8
Rhodotorula gracilis Rennerfelt[c]	5.6	2.8	1.4; <0.7
Saccharomyces cerevisiae Meyen	11.3	5.6	2.8

[a] Results based on 2 tests completed at different times; 2 figures are given where tests differed.

[b] Smallest amount of partly purified helixin (dry weight) that completely inhibited macroscopic growth.

[c] These organisms are yeasts not heretofore reported to be inhibited by helixin.

in a series of trials. Data for the tests are given in Table 2. These results indicated, as might be expected, that the antibiotic was more effective at pH 6.0 than at pH 4.7 or 3.0.

(b) *Resistance to washing by simulated rain.*—Resistance to washing by simulated rain of the partly purified helixin deposit on tomato leaves was studied using the washer previously described (4). Plants were sprayed with antibiotic preparation (8 units per ml.) at a given pH, dried, and leaf disk samples taken for assay. Plants were then washed, dried, and samples again taken for assay. It was not possible to prepare sprays of this potency more acid than pH 4.0 because a heavy gum formed that clogged the sprayer. A lower pH was obtained, however, by spraying plants first with antibiotic preparation and then, after they had dried, with a weak acid solution. By this procedure gum was formed on the leaves. Data for tests are given in Table 3. Deposits from the more alkaline sprays were readily washed from leaves; the antibiotic on the acid treated plants was resistant to washing.

TABLE 2.—*Early blight disease control by 45 μg. per ml. of partly purified helixin using sprays at different pH levels*

pH of spray	Trials[a]	Buffer[b]	Average disease control[c]
	No.		Percentage
3.0	4	+	70.7
4.7	3	+	77.4
6.0	3	+	96.3
6.0	7	—	97.2

[a] Each trial based on results from 3 test and 3 control plants. Trials completed at different times.

[b] A plus indicates that 0.01 M phosphate buffer was present. Minus sign indicates no buffer was added.

[c] Disease control for each treatment based on control plants sprayed with solution identical with test preparation except that antibiotic was omitted.

TABLE 3.—*The effect of the pH of the spray on the amount of helixin remaining on leaves after washing with simulated rain*

pH of spray	Cm. of rain	Percentage activity remaining after washing: Test 1	Test 2	Test 3
5.7	1.2	0	0	0
4.0	1.2	1	5	2
Below 4.0[a]	1.2	31	25	23
Below 4.0[a]	2.4	—	13	3

[a] Spray preparation at pH 4.0 applied to plants, plants dried, and then sprayed again with a weak acid solution (N/20 HCl in test 1 and N/40 H_2SO_4 in tests 2 and 3.

(c) *The stability on the leaf.*—Stability of helixin on tomato leaves was studied with the aid of the leaf disk assay, using plants that had been double-sprayed with antibiotic and N/40 H_2SO_4 as described above. Samples for assay were taken when the acid spray had dried and again at 2 and at 4 days. Three tests were made at different times. The average activity remaining after 2 days was found to be 61 per cent of the original; after 4 days the average activity remaining was 42 per cent of the original.

Studies on helixin for systemic action.—Two types of tests with *A. solani* on tomato plants were used to study possible systemic action of helixin: cutting tests, and whole-plant tests.

In cutting tests, stems of tomato cuttings 20–30 cm. high were placed in antibiotic solutions and allowed to absorb the solutions for 2 days in an artificially lighted room at 26°. They were then inoculated with *A. solani*, using methods cited above. Two tests were made; in each 4 or 5 replicate cuttings were placed in 3 concentrations of the antibiotic at pH 6.0 and in water, which served as a control. The antibiotic proved to be phytotoxic at concentrations of 7.5 μg. per ml. and greater, producing a yellowing of the lower leaves, a darkening of leaf interveinal areas, and a leaf flaccidity. At 7.5 μg. per ml. an average of 96 μg. was absorbed per cutting; however, this amount of antibiotic did not prevent infection by *A. solani*.

In whole plant tests, tomato seedlings were grown in white sand watered with Hoagland's solution until they were approximately 10 cm. high; they were then watered every other day for 10 days with 50 ml. of Hoagland's solution alone[5] or Hoagland's solution plus the antibiotic[5]. Plants were then inoculated with *A. solani* as described previously. Two tests, using 5 replicate plants for each of the 2 treatments,

[5] Solution adjusted to pH 6.0.

were made at different times: in 1 the antibiotic was used at 180 μg. per ml.; in the other it was used at 90 μg. per ml. At 180 μg. per ml. plant growth was retarded and the lower leaves yellowed; at the lower concentration no untoward effects were noted. However, the disease was not controlled by the application of helixin at either concentration.

Phytotoxicity studies.—The toxic effects of partly purified helixin on whole tomato plants and on tomato cuttings have been noted above. Additional information on the phytotoxicity of partly purified material at pH 6.0 was gained from a series of tests in which effects on foliage, germinating seed, and cuttings were observed. The foliage tests were made by spray application of 3 mg. per ml. of the antibiotic material on young bean, corn, cowpea, tomato, and wheat plants. No deleterious effects were observed in either of 2 tests on any of the plants up to 7 days, when the experiments were concluded. Petri plates containing filter paper moistened with the antibiotic solution were used for seed germination tests, the data for which were taken after 7 days at 24°. In 2 series of tests it was found that the germination of tomato seed was inhibited by 25 μg. per ml. of the antibiotic, cabbage and corn seed inhibited by 100 μg. per ml., and bean seed not inhibited by 200 μg. per ml. Cowpea cuttings at the first true leaf stage were used for the cutting tests: stems of cuttings were placed in solutions containing various concentrations of the antibiotic, and observations were made on the plants after 4 days in an artificially lighted room at 26°. Two series of tests at concentrations of 15 μg. per ml. and greater showed plants flaccid and growth and root development markedly retarded. These observations led to the conclusion that the partly purified antibiotic may be expected to be comparatively toxic to plants except when applied to the foliage.

Discussion.—The present study shows that helixin was an effective protectant fungicide in greenhouse tests with early blight disease, with lethal dose values in the same range as the most potent compounds that have come to our attention (7, 8). Present investigations employed partly purified helixin; it seems likely that when the pure material (or materials[6]) is isolated, even lower dosages will be inhibitory. In addition to the high activity of the impure material it was shown that under some conditions the antibiotic was comparatively stable on the leaf and was resistant to washing by simulated rain. Whether or not these properties of the antibiotic will remain or be enhanced once it is obtained in pure form cannot be determined without further investigation.

The present study shows that helixin was less active under acidic conditions, both when applied to the plant and *in vitro*. Studies on the effect of pH on the activity of antibiotics and bactericides are well known, but investigations of this sort dealing with the more modern plant fungicides have appeared rarely (2). This type of information would be of value from a fundamental point of view, and the knowledge obtained might well be of use in the control of plant diseases in the field.

[6] There is unpublished evidence at hand that partly purified preparations contain more than a single antibiotic.

SUMMARY

A partly purified preparation of helixin, an antifungal antibiotic, was tested in the greenhouse for plant disease protectant action by means of the tomato early blight technique. The LD50 and LD95 values were found to be 6.5 and 37.9 μg. per ml., respectively. The antibiotic was less toxic at pH 3.0 and 4.7 than under more alkaline conditions in agar-streak tests with 7 organisms and in early blight tests. It was shown by means of leaf disk assays that partly purified material under acidic conditions on the leaf was resistant to removal by simulated rain and was comparatively stable. No systemic action of the antibiotic was demonstrated. The partly purified material inhibited germination of the seeds of some plants at concentrations of 25–100 μg. per ml.; was toxic to tomato plants when applied to the roots at 180 μg. per ml.; and was phytotoxic to tomato and cowpea cuttings when absorbed at concentrations of 7.5–15 μg. per ml. and greater. No deleterious effects were noted when 3 mg. per ml. was applied as a spray to the foliage of several species of plants.

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A Simple Nephelometer.[1] J. K. RICHARDSON. In potato scab investigations, a study was made of the flora of soils in which different percentages of scab infection occurred on potato tubers. Nutritional studies of a large number of fungi, actinomycetes, and bacteria necessitated a rapid and accurate method of determining the amount of growth in a series of differential liquid media. Visual estimates of fungus and actinomycetous growth in test tubes were reasonably accurate and rapid, but the determination of variations in turbidity in bacterial cultures presented a much more difficult problem.

This was effectively solved by measuring turbidity photometrically by means of a simple apparatus (Fig. 1) constructed at the laboratory at small cost. A block of wood (C), 4 in. by 4 in. by 3 in., is attached by means of a metal collar to the lens tube (B) of a microscope lamp (A) equipped with a 100-watt projection bulb and adjusted in a horizontal position. Two parallel vertical holes are drilled in the block, 3 in. deep and 1 in. apart, with a diameter slightly greater than that of the test tubes to be used. An additional hole ¼ in. in diameter, and passing through the two vertical holes, extends horizontally, i.e., in line with the principal axis of the lens system. A shelf attached to the block below the ¼-in. hole on the side opposite to the light source supports a General Electric exposure meter, type DW-68 (D), in such a position that the light from the microscope lamp falls directly on the photoelectric cell. The light intensity is then measured directly in foot-candles.

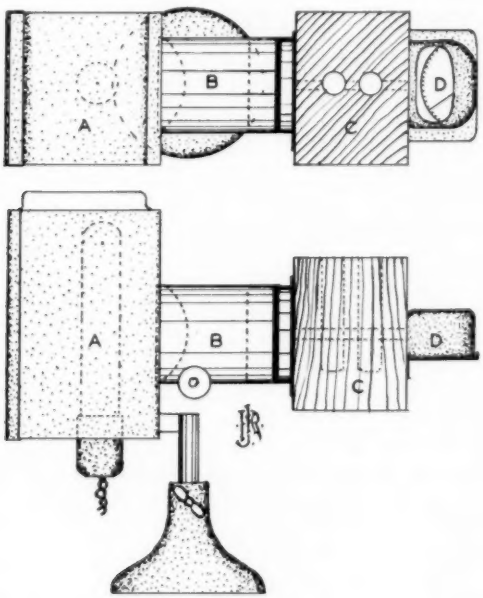

FIG. 1. A simple nephelometer, as viewed from above (top), and lateral view (bottom). A, microscope lamp with 100-watt projection bulb; B, lens tube; C, wooden block with metal shelf and collar attached; D, exposure meter.

[1]Contribution No. 1077 of the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, Canada.

In operating the nephelometer, a tube of noninoculated culture medium is used to obtain the standard no-growth reading in foot-candles. The meter readings taken through well agitated cultures then vary inversely with the turbidity of the medium, which is proportionate to the amount of growth. It is essential to use the same test-tube hole for all readings; the lens effect produced by a tube of medium gives a different meter reading with each distance from the light source.

The second test-tube hole is for the use of a compensating blank where turbidity in media of different colors is being recorded.

Variations of ½ foot-candle can be measured with this apparatus, whereas differences of 3 foot-candles are sometimes difficult to determine visually. This increase in accuracy, coupled with the ease and rapidity with which turbidity can be measured, makes this piece of equipment extremely valuable, particularly in making a large number of determinations.—Laboratory of Plant Pathology, St. Catharines, Ontario.

Phytotoxicity of Formaldehyde Residue on Flats. CHESTER N. ROISTACHER. It has long been known that when a formalin solution is allowed to evaporate it will polymerize to paraformaldehyde, a white powder, which volatilizes at a much slower rate than does the liquid. The literature on this subject has been reviewed by Hurd.[1] When a 5 per cent formalin solution was applied as a disinfectant to greenhouse flats, injury resulted to several kinds of seedlings grown in them. This injury was particularly noticeable in the outside rows of plants (Fig. 1). Tests were, therefore, made to determine the interval after spraying with formaldehyde before a flat could be considered safe to use with the most sensitive types of seedlings.

The test consisted of spraying flats with formaldehyde, keeping some dry and others wet, and testing at daily intervals for the degree of seedling injury. Petunia seedlings were used as the indicator plants because of their extreme sensitivity, and conditions of the test were such as to produce the maximum possible injury to the transplants by any toxic vapor present. Eleven redwood flats were thoroughly sprayed with a 5 per cent formalin solution and allowed to dry outdoors for 24 hr. After drying in the sun, five of the empty flats were placed in the greenhouse and watered twice daily; another five were kept dry. The

[1] Hurd, A. M. Injury to seed wheat resulting from drying after disinfection with formaldehyde. Jour. Agr. Res. [U.S.] 20: 209-244. 1920.

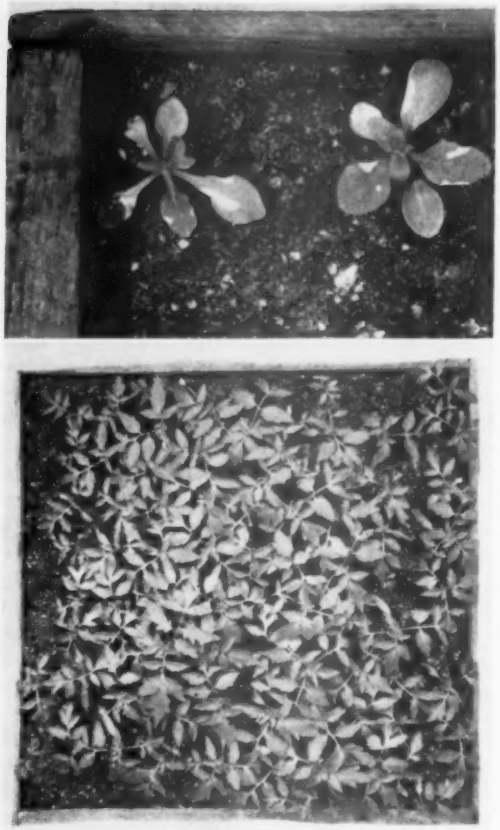

FIG. 1. Plant injury from the volatilization of paraformaldehyde. Top, injury to petunia transplanted to flats kept dry 4 days after the formaldehyde dip; bottom, marginal killing of tomatoes transplanted to flats kept dry 1 day after the formaldehyde dip.

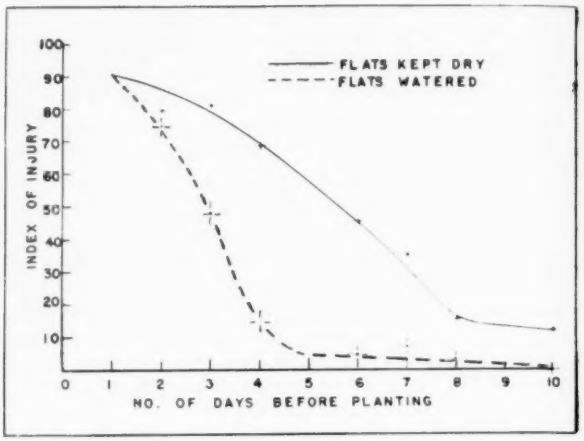

FIG. 2. Rate of volatilization of paraformaldehyde from flats kept moist or dry, as shown by the injury to petunia transplants.

remaining flat, not watered, was planted immediately. Each day one flat that had been watered, one flat that had been dry, and a steam-sterilized nontreated flat (check) were filled with a sandy loam to within $\frac{5}{8}$ in. of the top and 20 petunia seedlings were transplanted uniformly along the edges of each flat. They were then placed in separate chambers, each containing a 40-watt light bulb, and held at 80° F. ± 2° F. Each flat was covered with an inverted flat that had the bottom replaced with clear plastic. Thus air circulation was reduced to a minimum and enough light supplied to the plants to keep the stomata open. Under such conditions the plants would be subjected to any formaldehyde vapor present. All flats were watered lightly and then placed in the chambers; after 24 hr. they were removed to the greenhouse.

After 2 weeks in the greenhouse notes were taken on the degree of injury to each plant according to the following scale: 0, no injury; 1, slight burn on tip; 2, burn covering one-half to one-quarter of the leaf area; 3, entire leaf burned; 4, entire plant near death; 5, entire plant dead.

After each plant was rated, the total was taken as the index of injury caused by the toxic action of the volatilized paraformaldehyde. Since 20 plants were used per flat, a rating of 100 would indicate complete kill; 0 would indicate no injury. Figure 1 shows typical burn injury on petunias transplanted to flats that were held dry 4 days after the formaldehyde dip.

The averages for results of replicates are graphed in figure 2. The results show definite injurious effect from residual formaldehyde in the flats. Under the conditions of these tests, 4 or 5 days of watering were necessary to minimize the injury. Under normal greenhouse conditions 2 to 3 days of watering should be sufficient, depending upon other factors such as the concentration of the formaldehyde, the kind of plants grown, and the humidity and temperature between treating and planting. If the flats are held dry, 10 days or more should elapse before use.—Division of Plant Pathology, University of California, Los Angeles, California.